Part Four

PREPARATION OF RECOMBINANT VACCINIA VIRUS EXPRESSING FULL-LENGTH RABIES AND NON-RABIES LYSSAVIRUS GLYCOPROTEIN GENES AND EVALUATION OF IMMUNOGENICITY AND CROSS-PROTECTION IN A MOUSE MODEL

CHAPTER VII

TOWARDS A CROSS-PROTECTING LYSSAVIRUS VACCINE: IMMUNE RESPONSES AGAINST RECOMBINANT VACCINIA VIRUSES EXPRESSING FULL-LENGTH LYSSAVIRUS GLYCOPROTEINS

7.1 Introduction

The lyssaviruses belong to the family of *Rhabdoviridae* and order of *Mononegavirales* (*mono*-: single; *nega*-: indicating negative sense genome) (Fauquet *et al*., 2005; WHO Expert Consultation on Rabies, 2005). Based on sequence analysis of nucleo-, phospho- and glycoprotein genes, the lyssaviruses are currently divided into seven genotypes. These genotypes are recognized as genotype one, *Rabies virus*; genotype two, *Lagos Bat virus*, genotype three, *Mokola virus*, genotype four, *Duvenhage virus*, genotype five, *European Bat Lyssavirus-1*; genotype six, *European Bat Lyssavirus-2* and genotype seven, *Australian Bat Lyssavirus* (WHO Expert Consultation on Rabies, 2005). In addition, several novel lyssaviruses have been described in literature since 2003. These viruses were isolated from different bat species from central Asia and Russia and are called Aravan, Khujand, Irkut and West Caucasian Bat viruses (Arai *et al*., 2003; Botvinkin *et al*., 2003; Kuzmin *et al*., 2003). It has been suggested by the WHO Expert Consultation on Rabies that these four viruses should be recognized as separate genotypes based on current criteria for the taxonomy of lyssaviruses (WHO Expert Consultation on Rabies, 2005). In addition, the International Committee on the Taxonomy of Viruses has already recognized these viruses as putative species of the genus (Fauquet *et al*., 2005).

Considering phylogeny (comparison of glycoprotein sequences), immunogenicity and pathogenicity of isolates representing the range of lyssaviruses, Badrane and co-workers, recognized that the members of the genus could be grouped into two distinct phylogroups (Badrane *et al*., 2001). This division into phylogroups correlates with pattern of vaccine cross-protection observed for the lyssaviruses (Badrane *et al*., 2001; Nel, 2005). The first group is represented by isolates from genotype one, four, five, six and seven. The Irkut, Aravan and Khujand isolates also cluster with this group (Kuzmin

et al., 2005). Current vaccines and biologics, administered according to WHO prescribed regimens for pre- and post exposure, are typically effective against infections by viruses from this group (table 7.1). Vaccine failures are rare and have been reported when there have been deviation from the prescribed regimens and, or when the nature of the injuries of the victim have been especially severe (Haupt, 1999; Macgregor and Mehta, 2001; Southern and Eastern Africa Rabies Group, 2003; Sriaroon *et al*., 2005). On the other hand, the second phylogroup is represented by the African non-rabies lyssaviruses of genotype two and genotype three. Currently available vaccines and biologics fail to protect fully against infection with these viruses (table 7.1). Of particular interest, is one of the putative species before mentioned, the West Caucasian Bat virus (WCBV) isolate, which also groups with the latter phylogroup (Kuzmin *et al*., 2005). This virus was isolated from a common bent-winged bat (*Miniopterus schreibersi*) in Russia (Botvinkin *et al*., 2003). WCBV is recognized as the most phylogenetically divergent virus when compared on nucleotide and amino acid levels to the genotype one rabies viruses, and in addition only exhibits limited relatedness to genotype two and three viruses (Botvinkin *et al*., 2003; Kuzmin *et al*., 2005). Laboratory evidence also indicates little or no cross neutralization of anti-rabies sera with this isolate (Hanlon *et al*., 2005).

Limited attempts have been made towards developing biological products that protect against the viruses from phylogroup two. The studies that have been geared towards this goal have employed the expression of Mokola virus glycoprotein genes. The Mokola virus has been recognized as the most phylogenetically divergent lyssavirus until recently with the description of the WCBV. Mokola virus glycoprotein has been expressed in the baculovirus system (Tordo *et al*., 1993). The protein produced in the insect cells proved immunogenic, and protected against lethal Mokola virus challenge in a mouse model. Protection was however not afforded against challenge with rabies virus. Mokola virus glycoprotein genes have also been expressed in different DNA vaccine carriers and the immunogenicity of the constructs compared (Nel *et al*., 2003). In this study, protection against Mokola virus challenge could be afforded upon booster administrations of the recombinant genetic vaccines. Once again the Mokola virus antigen expressing subunit vaccines did not afford protection against rabies virus

Table 7.1 Summary of available reports on the cross-protective efficacy and cross-reactivity of rabies commercial and experimental vaccines and immunoglobulin.

VIRUS OR GROUP OF VIRUSES	CROSS-PROTECTION AND CROSS-REACTIVITY	REFERENCE
Genotype 1, rabies viruses	Commercial tissue culture vaccines are fully protective against a spectrum of genotype one isolates[1, 2, and 3]. Human RIG cross neutralizes an array of genotype one isolates[3, 4]. Vaccinia virus expressing a glycoprotein gene or both a glycoprotein gene and a nucleoprotein gene protected against a selection of different genotype 1 isolates[2]. Mokola virus ribonucleoprotein protected against peripheral challenge with CVS in mice[5]. Mice were protected against challenge with CVS upon pre-exposure vaccination with Pasteur, Pittman-Moore or Flury Low egg Passage- based vaccines[6].	Koprowski *et al*., 1985 (1) Lodmell *et al*., 1995 (2) Brookes *et al*., 2005b (3) Hanlon *et al*., 2001 (4) Dietzschold *et al*., 1987 (5) Lafon *et al*., 1988 (6)
Genotype 2, Lagos Bat viruses	HDCV vaccinated mice were not protected against lethal challenge with Lagos Bat virus[1]. Human RIG fails to cross neutralize the virus[2]. Sera from vaccinated humans failed significantly cross neutralize the virus[3].	Fekadu *et al*., 1988 (1) Hanlon *et al*., 2001 (2) Hanlon *et al*., 2005 (3)
Genotype 3, Mokola viruses	Mice that received HDCV pre-exposure were not protected against lethal Mokola virus challenge[1]. Human RIG failed to cross neutralize a Mokola virus isolate[2]. Serum form vaccinated individuals to significantly cross-react to Mokola virus[3].	Fekadu *et al*., 1988 (1) Hanlon *et al*., 2001 (2) Hanlon *et al*., 2005
Genotype 4, Duvenhage viruses	HDCV vaccinated mice were protected against lethal challenge with Duvenhage virus[1]. Cross-reactivity of the virus and human RIG was reported[2].	Fekadu *et al*., 1988 (1) Hanlon *et al*., 2001 (2)
Genotype 5, European Bat Lyssavirus-1 (EBLV-1)	No protection was afforded upon pre-exposure vaccination with Pittman-Moore strain or Flury Low Egg Passage strain based vaccines in mice after intracranial challenge with EBLV-1 upon intracranial challenge[1, 2]. HDCV did however protect mice against lethal peripheral challenge with EBLV-1[2]. Pasteur virus based vaccine however offered noteworthy protection in mice in the same experiment[1]. DNA vaccines expressing Pasteur virus glycorprotein reportedly induced humoral responses that cross-neutralized EBLV-1, but the levels were low[4]. HDCV vaccinated mice were protected from lethal peripheral challenge[2]. Montano-Hirose and co-workers (1993) reported the lack of significant protection was afforded after human or equine RIG administration against lethal intramuscular EBLV-1 challenge in mice[3].	Lafon *et al*., 1988 (1) Brookes *et al*., 2005b (2) Montano-Hirose *et al*., 1993 (3) Perrin *et al*., 2000 (4)
Genotype 6, European Bat Lyssavirus-2	HDCV vaccinated mice were protected against lethal peripheral and intracranial challenge[1]. Human RIG cross neutralized the virus[2]. DNA vaccines expressing Pastuer virus glycoprotein gene induced humoral responses that cross-neutralized EBLV-2 significantly (3).	Brookes *et al*., 2005b (1) Hanlon *et al*., 2001 (2) Perrin *et al*., 2000 (3)
Genotype 7, Australian Bat Lyssavirus	Mice were protected from lethal peripheral challenge with Australian bat lyssavirus after vaccination with HDCV. The vaccine did however not offer statistically significant protection against intracranial challenge[1]. Cross-neutralization has been reported with human RIG[2].	Brookes et al.,2005b (1) Hanlon *et al*., 2001 (2)
Aravan, Irkut and Khunjand virus isolates	Differential protection against Aravan, Irkut and Khunjand virus challenge in hamsters after pre-exposure vaccination with HDCV, a commercial veterinary vaccine or V-RG have been noted. All three vaccines protected significantly against challenge with Khunjand virus, whilst only the veterinary vaccine offered considerable protection against Aravan virus challenge. Post-exposure regimens employing human RIG, equine RIG and/or HDCV failed to protect hamsters from lethal challenge with Irkut virus. Statistically insignificant survivorship in hamsters was noted upon challenge with Aravan and Khunjand viruses.	Hanlon *et al*., 2005
West Caucasian bat virus virus isolate	No protection conferred in hamsters upon pre-exposure vaccination with HDCV, a commercial veterinary vaccine or V-RG. Equine RIG and human RIG failed to cross neutralize the virus. Sera from vaccinated humans were not able to cross-neutralize the virus.	Hanlon *et al*., 2005

challenge. In another study, chimeric lyssavirus glycoprotein genes were constructed (Jallet *et al*., 1999). These genes carried critical glycoprotein antigenic sites from both the rabies.virus and the Mokola virus glycoprotein genes. The genes were expressed in a DNA vaccine model and afforded protection 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). The sera significantly cross neutralized Lagos Bat virus, European Bat Lyssavirus-2, but only partially cross neutralized Duvenhage and European Bat Lyssavirus-1 viruses (Bahloul *et al*., 1998).

Studies towards the broadening of the vaccine cross protection spectrum have also focused on investigating the role of the nucleoprotein in subunit vaccines. The rationale for these studies is based on the observed high level of conservation of the nucleoprotein sequences among the lyssaviruses (Bourhy *et al*., 1993). On amino acid level there is a 77% similarity between the nucleoprotein of the seven genotypes. In addition the nucleoprotein carries various epitopes, including T helper cell epitopes (Goto *et al*., 1995). The findings in these studies profess contrasting findings, and more research on the topic is warranted. Rabies virus ribonucleoprotein administered in complete Fruend's adjuvant protected against peripheral challenge with rabies and Mokola viruses (Dietzschold *et al*., 1987b). Likewise, Mokola virus nucleoprotein administered in complete Fruend's adjuvant protected against peripheral challenge with rabies and Mokola viruses. Recombinant vaccinia viruses expressing the rabies virus glycoprotein or both a glycoprotein and nucleoprotein gene protected equally against lethal challenge with rabies viruses (Lodmell *et al*., 1995). In a back titration of the vaccines a synergistic effect of the dual antigen expressing vaccine could not be shown. Rabies virus glycoprotein and nucleoprotein were immunogenically expressed by *Autographa californica* polyhedrosis virus in *Spodoptera frugiperda* or *Trichoplusia ni* cell culture (Drings *et al*., 1999). Although the inclusion of nucleoprotein in the vaccine did not enhance the virus neutralizing responses elicited by the glycoprotein vaccine, a so-called adjuvant effect was noted. The virus neutralizing responses elicited when the nucleoprotein and glycoprotein were co-administered, however, did not enhance the cross neutralization of a European Bat Lyssavirus-1 isolate. In another study, DNA vaccines

expressing both the Mokola virus glycoprotein gene and nucleoprotein gene did not enhance either the protection against Mokola virus infection or the cross-protection ability of the vaccine (Nel *et al*., 2003)

The aim of this study was to determine the cross-protective value of single and dual antigen expressing vaccinia virus vectored vaccines. These vaccines were designed to express the rabies, the WCB or the Mokola virus glycoprotein genes, in single or in dual combinations of the genes.

7.2 Materials and methods

7.2.1 Viruses, plasmids and genes

Vaccinia virus Copenhagen strain was obtained from Dr. C.E. Rupprecht (Centers for Disease Control and Prevention (CDC), United States of America). A partially prepared recombinant vaccinia virus encoding a rabies virus glycoprotein gene of the Evelyn-Rockitniki-Abelseth (ERA) strain of rabies virus was also obtained form Dr. C.E. Rupprecht. The gene was inserted into the hemagluttinin gene (HA) of the vaccinia virus genome and carries a neomycin selection marker gene (Dvoracek and Shors, 2003). A recombinant vaccinia virus transfer vector, pGVWR-RG previously constructed was also used in this study[26]. Dr. I.V. Kuzmin (CDC, USA) provided hyper immune mouse anti-WCBV serum for the detection of expression of the WCBV glycoprotein. A mouse monoclonal antibody against Mokola virus glycoprotein (1409-7) was provided by M. Niezgoda (CDC, USA). The mouse monoclonal antibodies M778 and M725 (directed to antigenic site II and III, respectively) were used for the expression analysis of the rabies virus glycoprotein gene expression analysis, was a generous gift from Dr. A.I. Wandeler (Center of Expertise for Rabies, Canadian Food Inspection Agency, Canada).

Challenge viruses for the experiments as follows. Suckling mouse brain homogenates of a Mokola virus isolate (Agricultural Research Council-Onderstepoort Veterinary Institute (ARC-OVI) reference number: RA361) made from a domestic cat in Pietermaritzburg, KwaZulu-Natal, South Africa were prepared and provided by M. Niezgoda. Three WCBV positive suckling mouse brains were provided by Dr. I.V.

[26] Refer to Part Three, Chapter V, section 5.2.4

Kuzmin. A southern African strain of mongoose rabies virus (ARC-OVI reference number: m710/90) was obtained from Dr. C.T. Sabeta and grown and tittered as previously described[27]. Rabies virus (CVS-11 strain) and Mokola virus (ARC-OVI reference number: RA361) challenge viruses for the rapid fluorescent foci inhibition test (RFFIT) were obtained from M. Niezgoda. Cell culture-adapted WCBV and Lagos Bat virus (a Senegal isolate) challenge viruses for the RFFIT was obtained from W. Weldon (CDC, USA), and W. Markotter (University of Pretoria, South Africa), respectively.

The construction of a vaccinia virus transfer vector, pGWR-gptNew was described elsewhere[28]. Complementary DNA encoding the WCBV glycoprotein gene was provided by Dr. I.V. Kuzmin. The Mokola virus glycoprotein gene was obtained from a recombinant construct, pBudCE4-MokG provided by Prof. L.H. Nel (University of Pretoria, South Africa). The particulars of the Mokola virus glycoprotein recombinant are described in Nel *et al*., 2003.

7.2.2 Animals used in the study

ICR mice (female, three weeks and older) and pregnant mice (for suckling mice) were obtained from Harlan Sprague Daly (USA). Animals were housed and handled according to protocols approved by the Institutional Animal Care and Use Committee of the Centers for Disease Control and Prevention.

7.2.3 Molecular cloning of the WCBV glycoprotein gene

7.2.3.1 Polymerase Chain Reaction (PCR) of the WCBV glycoprotein gene and cloning in the PCR cloning vector, pGEM-T Easy®

The primers, WCBV G forward (CTCATCTCAGAGAAATGGC) and WCBV G reverse (CCCTTGAAGAATTCAATACC) were designed and used for the PCR amplification of the WCBV glycoprotein gene.

A PCR master mix was prepared containing for each reaction:

[27] Refer to Part Three, Chapter VI, section 6.2.2
[28] Refer to Part Three, Chapter V, section 5.2.4.1

1X ExTaq Polymerase buffer (Takara Biochemicals)
10 mM dNTP mixture (2.5 mM of each dNTP; Takara Biochemicals)
10 pmol of each primer
1 unit of ExTaq polymerase (Takara Biochemicals)

5 µl of the WCBV glycoprotein gene complimentary DNA[29] was used as template and the volume of the reagents were adjusted to 50 µl per reaction with nuclease free water. The PCR cycle parameters used as follows: 90 seconds at 94 ˚C, followed by 30 cycles of 30 seconds at 94 ˚C, 40 seconds at 55 ˚C and 90 seconds at 72 ˚C, and finally a termination cycle of 7 minutes at 72 ˚C. The PCR product was resolved on a 0.8% agarose gel prepared with Tris-Borate-Ethylenediaminetetraacetic acid buffer (TBE) (45 mM Tris-borate, 1 mM EDTA, pH8.0) and stained with 0.5 µg/ml ethidium bromide (Promega). The PCR amplicon's size was verified against a molecular weight marker. The amplicon was purified using the Wizard® PCR DNA Clean-Up system (Promega) according to the centrifugation protocol suggested by the manufacturer.

The purified PCR product was cloned into the pGEM-T Easy® vector (Promega) (figure 7.1) following the manufacturer's protocol and a insert: vector molecular weight ratio of 3:1. A rapid ligation protocol was followed using a rapid ligation buffer for T4 DNA ligase (New England Biolabs). The ligation reaction was transformed in competent JM109 cells (High efficiency competent cells, transformation efficiency of 10^8, Promega), following a heat shock protocol per the supplier's protocol. The transformed cells were plated onto agar plates with 100 µg/ml ampicillin. In addition, 10 mM isopropyl β-D-1-thiogalactopyranoside (Sigma Aldrich) and 40 µl 2% 5-Bromo-4-chloro-3-indolyl-β-D-galactopyronoside (Promega) was plated out with the cells per plate to facilitate blue-white selection. The plates were incubated overnight at 37 ˚C. Subsequently white colonies (and blue colonies as control) were picked and cultured in Luria Bertani (LB) broth (1% tryptone, 1% sodium chloride, 0.5 % yeast; pH 7.4) containing 100 µg/ml ampicillin antibiotic while shaking at 230 rates per minute at 37 ˚C. Plasmids were screened according to a rapid nucleic acid isolation protocol by Beuken *et al*., 1998. Possible recombinants were identified and further analysed by QIAprep Spin

[29] Refer to section 7.2.1

miniprep kit (QIAGEN) and restriction enzyme digestion. The recombinant clones were named pGEM-WCBVG. The WCBV glycoprotein gene of recombinant clones was sequenced using M13 universal primers (Promega). The sequencing reactions were prepared with the Applied Biosystems BigDye® terminator kit as prescribed by the manufacturer. The sequences were run on an ABI 377 gel sequencer machine at the Rabies Section of the CDC, USA. The sequences were examined for PCR incorporated errors and blasted against available sequence on NCBI GENBANK.

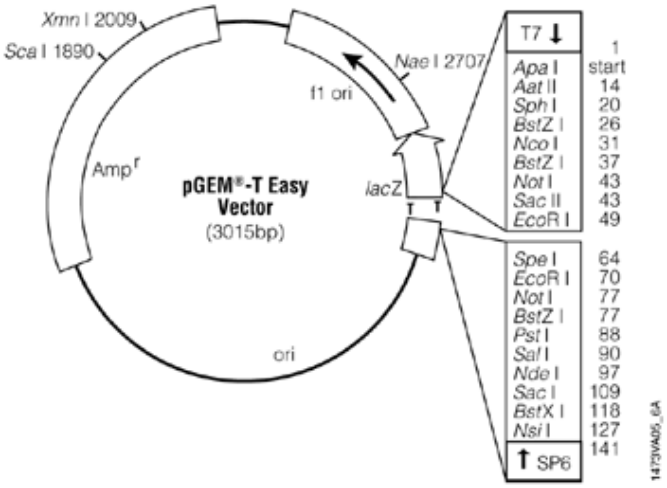


Figure 7.1: Plasmid map of the PCR cloning vector, pGEM-T Easy® (Internet reference 6).

7.2.3.2 Cloning of the WCBV glycoprotein gene in a vaccinia virus transfer vector, pGVWR-gptNew

The glycoprotein gene was digested from pGEM-WCBVG with *Eco*R I enzyme (Promega) and inserted into the *Eco*R I site of the mammalian expression vector, pCINeo (Promega). The gene was then retrieved by digestion with *Xho* I (New England Biolabs) and *Sal* I (New England Biolabs) in an compatible buffer system and inserted into the *Sal* I site of pGVWR-gptNew (figure 7.2). The direction of the insertion was determined by digestion with *Eco*R V restriction enzyme (New England Biolabs). The same general molecular cloning techniques were used as described elsewhere[30].

[30] refer to 7.2.3.1

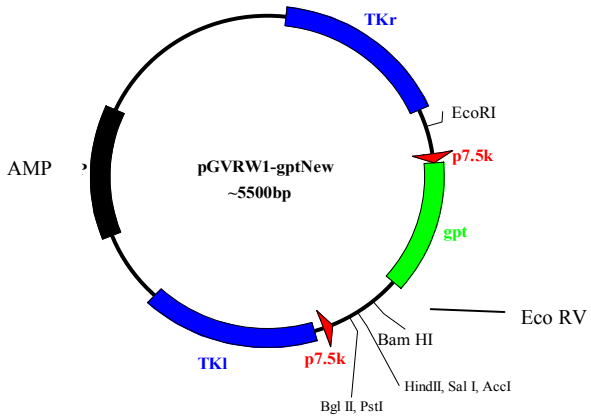


Figure 7.2: Plasmid map of the vaccinia transfer vector, pGVWR-gptNew. The Tkr and Tkl flanking regions enclose an expression cassette with a p7.5K early-late vaccinia virus promoter region. The selectable marker gene *Eco*gpt is also under regulation of the p7.5K.

7.2.4 Molecular cloning of a Mokola virus glycoprotein gene in pGVWR-gptNew

Molecular cloning techniques employed were discussed before[30]. pBudCE4-MokG plasmid was transformed in competent JM109 cells (High efficiency competent cells, transformation efficiency of 10^8, Promega), following a heat shock protocol as suggested by the manufacturer. The transformation reactions were plated onto LB agar plates containing zeocin at 50 µg/ml. Following overnight incubation at 37 ˚C, single colonies were picked and cultured in LB broth containing zeocin. Plasmids were isolated with a QIAprep Spin miniprep kit (QIAGEN), according to the manufacturer's suggestions.

The Mokola virus glycoprotein gene was digested for pBUDCE4-MokG with *Pst* I (New England Biolabs) according to the manufacturer's suggestions. The purified DNA was inserted into the *Pst I* site of pGVWR-gptNew. The ligation reaction was transformed and the clones screened as previously described, but without the addition of the reagents for blue-and-white-selection[30]. The direction of the insertion was determined by digestion with *Bam*H I and *Eco*R V restriction endonuclease (New England Biolabs). Recombinant plasmids with the gene in the correct orientation were given the name pGVWR-MokG.

7.2.5 Generation and isolation of recombinant vaccinia viruses expressing lyssavirus glycoprotein genes

7.2.5.1 Purification of a recombinant vaccinia virus Copenhagen encoding a rabies virus glycoprotein gene

Vero cell culture (African green monkey cell line, American Type Culture Collection reference number, CCL-81) was maintained in minimum essential medium (MEM) supplemented 4 mM glutamine, 2 X MEM vitamin solution, and 0.75 mg/ml antibiotics (penicillin, 100 units/ml; streptomycin, 100 µg/ml and amphotericin B, 0.25 µg/ml) (all from Invitrogen Life Technologies, Gibco). The cultures were incubated at 37 ˚C and 0.5 % CO_2.

The recombinant virus isolate was passaged under G418 resistance selection in order to purify the isolate from wild type virus background. Briefly, the virus was harvested by freeze-thawing three times the provided cell culture soup, and collecting the supernatant after a brief centrifugation at 200 *g* in a benchtop microcentrifuge. The supernatant was diluted in ten fold serial dilution and titrated unto confluent monolayers of Vero cell culture. The cells were fed with cell culture medium containing 1 mg/ml G418 antibiotic (Invitrogen Life Technologies, Gibco). The infected cultures were incubated for 48 hours at 37 ˚C and 0.5 % CO_2. The spent cell culture medium was removed and the monolayers washed with phosphate buffered saline (PBS) (13.7 mM NaCl, 0.27 mM KCl, 0.43 mM $Na_2HPO_4.2H_2O$, 0.14 mM KH_2PO_4, pH 7.4). Plaques were picked with sterile barrier tips from the wells with single plaques. The plaque purification procedure was repeated for five more rounds. The resultant recombinant virus clones were named VV-RG. The homogeneity of the recombinant virus isolates were monitored with a PCR designed for the HA gene region of the vaccinia virus Copenhagen gene. The particulars of this procedure are described in section 7.2.5.4.

7.2.5.2 Transfection for the generation of recombinant vaccinia viruses

Monolayers of Vero cells were prepared in 12 well cell culture plates (Corning). Confluent monolayers were infected with vaccinia virus Copenhagen strain (for the generation of single gene constructs) or VV-RG (for the generation of double gene constructs) at a multiplicity of infection of 0.01 (table 7.2). The virus was absorbed for 90 minutes at 37 ˚C and 0.5 % CO_2. Transfection complexes were prepared with the activated-dendrimer transfection reagent, Superfect® (QIAGEN) and the recombinant transfer plasmids, pGVWR-WCBVG, pGVWR-RG and pGVWR-MG. The standard protocol for the stable transfection of adherent cell cultures was followed as suggested by the manufacturer. The transfection complexes were added to the cells and incubated at 37 ˚C and 0.5 % CO_2 for 48 hours.

Table 7.2: Description of the recombinant vaccinia virus vaccines generated and tested in this study

RECOMBINANT VACCINE VIRUS (ABBREVIATED NAME HEREAFTER USED)	**PARENT VIRUS**	**TRANSFER PLASMID**	**BRIEF DESCRIPTION OF VIRUS**
Vaccinia-rabies virus glycoprotein **(VV-RG)**	Vaccinia Copenhagen	PBRExpress-rabies glycoprotein	Glycoprotein of ERA rabies virus inserted into HA gene. Carries neomycin resistance marker.
Vaccinia-Mokola virus glycoprotein **(VV-MG)**	Vaccinia Copenhagen	pGVWR-MG	Mokola virus glycoprotein inserted into TK gene of vaccinia virus Copenhagen. The virus encodes a mycophenolic acid resistance marker.
Vaccinia-West Caucasian bat virus glycoprotein **(VV-WG)**	Vaccinia Copenhagen	pGVWR-WG	WCBV glycoprotein inserted into TK gene of vaccinia virus Copenhagen. The virus encodes a mycophenolic acid resistance marker.
Double rabies virus glycoprotein expressing vaccinia **(VV-RGRG)**	VV-RG	pGVWR-RG	Glycoprotein gene of ERA rabies virus inserted HA gene and glycoprotein gene of mongoose virus isolate inserted into TK gene. Encodes both neomycin and mycophenolic acid resistance marker genes.
Vaccinia-rabies virus-Mokola virus glycoprotein **(VV-RGMG)**	VV-RG	pGVWR-MG	Glycoprotein gene of ERA rabies virus inserted HA gene and Mokola virus glycoprotein gene inserted into TK gene. Encodes both neomycin and mycophenolic acid resistance marker genes.
Vaccinia-rabies WCB virus glycoprotein **(VV-RGWG)**	VV-RG	pGVWR-WG	Glycoprotein gene of ERA rabies virus inserted HA gene and Mokola virus glycoprotein gene inserted into TK gene. Encodes both neomycin and mycophenolic acid resistance marker genes.

7.2.5.3 Purification and isolation of recombinant vaccinia viruses

The transfection wells were harvested by freeze thawing three times followed by a brief centrifugation at 200 *g* to collect cellular debris. The harvested supernatants were diluted in ten fold serial dilutions and titrated on monolayers of Vero cells. The viruses were incubated (37 ˚C and 0.5 % CO_2) under mycophenolic acid (MPA) resistance selection. The selection medium was prepared by adding 25 µg/ml MPA, 250 µg/ml xanthine and 15 µg/ml hypoxanthine (all from Sigma-Aldrich) to the growth medium. After 48 hours of incubation the cell culture medium on the infections were removed and the monolayers washed with PBS. Distinctly single plaques were picked from the wells of the highest dilution showing cytopathic effect. The viruses were harvested, titrated and incubated under selection as before. The plaque purification procedure was repeated for 5 more rounds. The homogeneity of the recombinant isolates were analysed with PCR of the thymidine kinase (TK) region of the vaccinia virus Copenhagen genome, as described in the next section, 7.2.5.4.

7.2.5.4 Analysis of homogeneity of recombinant vaccinia virus clones: PCR of the site of HA and TK gene regions of the vaccinia virus Copenhagen genome

Genomic DNA was extracted from virus isolates from subsequent passages as described by Esposito *et al*., 1981 and as previously discussed[31]. Primers that anneal in the regions that flank the HA gene of the vaccinia virus genome was designed, HA+ (GACACGATTACCAATACTTTTG) and HA-(CTTCTTTATCAGTAATTGGTTC), and regions that flank the TK gene, VacTKl (TCCCATCGAGTGCGGCTAC) and VacTKr (GTCCCATCGAGTGCGGCTAC). PCR for both these regions was preformed for each isolate. A PCR master mix for each primer set was prepared as follows:

1X ExTaq Polymerase buffer (Takara Biochemicals)
10 mM dNTP mixture (2.5 mM of each dNTP)
10 pmol of each primer (HA+ and HA-, or VacTKl and VacTKr)
1 unit of ExTaq polymerase (Takara Biochemicals)

[31] Refer to Part Two, Chapter III, section 3.2.5

5 µl of the extracted genomic DNA was used per reaction, and the volume of each PCR adjusted to 50 µl with nuclease free H_2O. A negative control, without the addition of a template preparation, was also included. Positive controls were included using a recombinant plasmid and wild type virus genomic DNA as templates. The PCR cycle as follows: denaturation step of 90 seconds at 94 ˚C followed by 35 cycles of 30 seconds at 94 ˚C, 40 seconds at 55 ˚C and 3 minutes at 72 ˚C. The cycle was completed with a 7 minute step at 72 ˚C. The PCR products were analysed on 0.8 % TBE agarose gels stained with 0.5 µg/ml ethidium bromide.

7.2.5.5 Expression analysis: indirect immunofluoresence assay

Vero cell monolayers were prepared in Lab-Tek 8 well chamber slides (Nalge Nunc International). Confluent monolayers were infected with the respective recombinant viruses. Controls were prepared by infection with vaccinia virus Copenhagen strain and including uninfected cell control. Additional controls were included to test for the cross-reactivity of the primary antibodies with heterologous glycoproteins. After 48 hours of incubation cell culture supernatants were removed and the cells fixed with ice-cold acetone for 10 minutes. Mouse hyper immune serum was used to test for the expression of WCBV glycoprotein. The antibody was previously titrated against WCBV infected cell culture and optimal dilution ratio estimated. Mouse monoclonals against Mokola and rabies virus glycoprotein, respectively, were used to test for the expression of the Mokola and rabies virus glycoproteins. The dilution ratios for these antibodies were also determined likewise. The fixed cultures were flooded with the primary antibody and incubated at 37 ˚C for 60 minutes. Incubation was followed by four, ten minute washes in PBS with gentle rocking. Fluorescein isothiocyanate (FITC) labeled, anti-mouse IgG (Sigma Aldrich, Cat. No. F5897) was diluted 1:64 (as suggested by the manufacturer) in blocking solution (1% fat-free milk) with 0.01% Evan's Blue and was transferred to the fixed cells and incubated for 30 minutes at 37 ºC. The incubation was followed by four, ten minute washes with PBS and a final ten-minute wash in distilled H_2O. Preparations were air dried, mounted in non-permanent mounting fluid

(50% glycerol, 50% PBS, pH 7.4) and visualized with a fluorescence microscope (Axioscope, Zeiss) and images captured with digital camera.

7.2.6 Preparation of the vaccine viruses for immunization study

7.2.6.1 Large scale growth and semi-purification from single, plaque isolates

The recombinant vaccine viruses and vaccinia Copenhagen were cultured on Vero cell culture and purified as previously described[32].

7.2.6.2 Titration

The viruses were tittered in a plaque assay and the plaque forming units per milliliter (pfu/ml) determined as described before[33].

7.2.7 Preparation of WCBV challenge material

7.2.7.1 Amplification and preparation of WCBV suckling mouse brain homogenates

Three WCBV positive suckling mouse brains were homogenized in PBS with 2% horse serum. Briefly, the brains were weighed and pooled to prepare 20 % (w/v) suspensions. After the brain material was macerated in a chilled glass mortar and pestle, the suspensions were centrifuged at 200 *g* and 4 ˚C for 10 minutes. The supernatants were collected, aliquoted and stored at -80 ˚C. Two to three day old suckling mice received 30 µl of the suckling mouse brain suspension intracranially, with a 0.5cc tuberculin syringe and 8 mm, 31 gauge needle (Becton Dickinson and Company, USA). Animals were monitored for up to 21 days after inoculation and either killed by euthanasia or collected upon death. The brain material of the dead animals was removed aseptically. The direct fluorescent antibody test (dFA) was used for postmortem

[32] Refer to Part Three, Chapter V, section 5.2.9
[33] Refer to Part Three, Chapter V, section 5.2.10.2

diagnosis of rabies infection in the animals[34]. The brain material that tested positive by dFA was pooled and used to prepare 20% (w/v) homogenates as before.

7.2.7.2 Titration of suckling mouse brain homogenates

The protocol was adapted from Koprowski, 1994. Titrations were carried out in 3-4 week old ICR mice. The virus homogenates were diluted in a ten fold serial dilution in the range of 10^{0} to 10^{-8} in PBS with 2% horse serum. Animals received 30 µl of homogenate intracranially with a 0.5cc tuberculin syringe and 8 mm, 31 gauge needles (Becton Dickinson and Company, USA). Animals were observed for up to 21 days after inoculation and either killed by euthanasia by cervical dislocation when required or collected upon death. The direct fluorescent antibody test (dFA) was used for postmortem diagnosis of rabies infection in the animals[35]. The mouse intracranial lethal dose 50 ($MICLD_{50}$) titers were subsequently calculated from the data according to Reed and Muench's method for the determination of 50 % endpoints (Reed and Muench, 1938).

7.2.8 Immunization and challenge study

Six week old ICR mice were divided into groups of 30 mice each, except for the mock control groups that only had 15 mice each the group. Each group of 30 mice received a particular recombinant vaccine. The mice received 10^{7} pfu of the vaccine viruses or their parent viruses in 50 µl of MEM with 1 X antibiotic. The administrations were made intramuscularly in the right quadriceps muscle with a 0.5cc tuberculin syringe with an 8mm, 31-gauge needle (Becton Dickinson and Company, USA). All the animals received a booster of 10^{5} pfu of the same vaccine virus or parent virus on 14 days after the primary immunization. The administrations were similar to that of the primary immunizations. Mock controls were included by immunizing mice with only MEM preparations.

Blood was collected *via* the retro-orbital route on day 0, day 7 and day 21. Blood was collected using heparinized microhematocrit capillary tubes (Becton Dickinson and

[34] Refer to Part Three, Chapter VI, section 6.2.3.3

Company, USA) or heparinized Natalson blood collecting capillaries (Fisher Scientific, USA). Sera were separated in Microtainer® serum separation tubes with SST™ (Becton Dickinson and Company, USA) as suggested by the manufacturer, and stored at – 20 ˚C until analysis.

On day 28 the groups of 30 animals were divided into three groups of 10 animals each, which then received lethal intracranial challenge with rabies virus, Mokola virus or WCBV. The inoculations were in volumes of 30 µl each and were done with 0.5cc tuberculin syringe with an 8mm, 31-gauge needle or 1 cc syringes with 27-gauge needles (Becton Dickinson and Company, USA). The animals were monitored for up to 21 days after the challenge. Animals were killed by euthanasia when required or collected upon death. Post mortem diagnosis of rabies virus infection was done by dFA as before[35].

7.2.9 Analysis of humoral responses: RFFIT

The preparation and interpretation of the RFFIT is described elsewhere[36]. In modification of this protocol challenge virus included not only the challenge virus standard (CVS-11), but also Mokola virus, Lagos Bat virus and WCBV. These viruses were all diluted to 50 fluorescent foci doses per 100 µl before use.

7.2.10 Statistical analysis of data

The one-tailed analysis of variance between groups (ANOVA) and student's t test were used for the analysis of data. ANOVA was used for comparisons between multiple groups, whereas the student's t test was used for comparison between two groups. ANOVA tests were calculated online (www.physics.csbsju.edu/stats/anova.html, internet reference 3). A confidence level of $p > 0.05$ was assumed for both tests.

[35] Refer to Part Three, Chapter VI, section 6.2.3.3
[36] Refer to Part Two, Chapter IV, section 4.2.3

7.3 Results

7.3.1 Molecular cloning of WCBV glycoprotein gene in a vaccinia virus transfer vector

The PCR primers, WCBV G forward and WCBV G reverse, were used to amplify a region of about 1 600 base pairs which included the WCBV glycoprotein gene (figure 7.3). The PCR product was cloned into the PCR cloning vector, pGEM-T Easy® and sequenced using (universal primers that bind within the plasmid vector) to determine the integrity of the amplified product (results not shown). Clones with open reading frames and 100 % sequence homology to the WCBV glycoprotein gene on the GENBANK database were identified for use in subsequent cloning steps. The gene was subcloned in the mammalian expression vector, pCINeo (results not shown). The gene was then inserted into the *Sal* I site of the transfer vector, pGVWR-gptNew (figure 7.4). Recombinant plasmids were identified by digestion with *Sal* I, which yielded a DNA band of approximately 7 100 base pairs (compare with DNA band of about 5 500 base pairs for the non-recombinant plasmid). The direction of the insertion of the gene relative to the promoter was determined by digestion with *Eco*R V restriction endonuclease, which yielded DNA bands of 500 and 6 600 base pairs for clones with the gene in the correct orientation, and, bands of 1 600 and 5 500 base pairs for clones with the gene in the incorrect orientation.

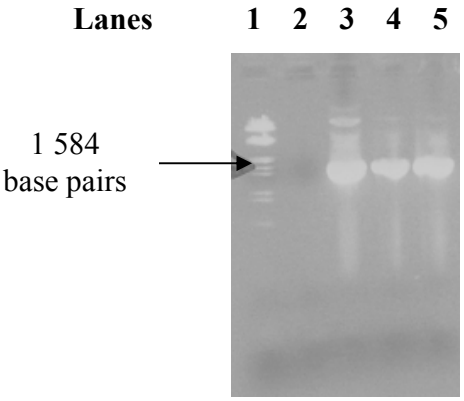


Figure 7.3: PCR of the WCBV glycoprotein gene. PCR was preformed on increasing dilutions of complimentary DNA template (lanes 3-5). The amplicons were run against a molecular weight

marker (lambda DNA *Eco* RI/*Hin*d III marker, Promega) (lane 1). The negative control PCR (only H_2O as templates) was resolved in lane 2.

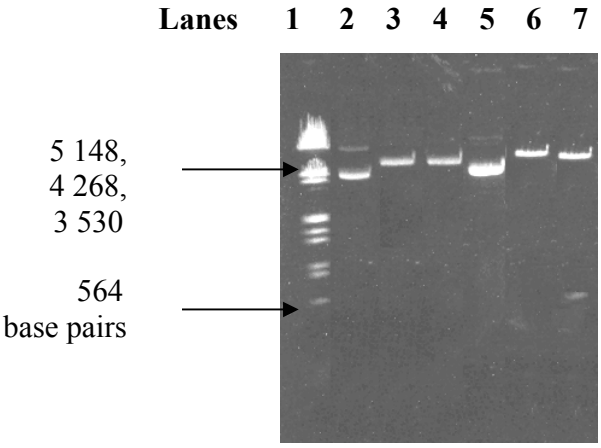


Figure 7.4: Electrophoretic profile of a pGVWR-WG clone with the gene in the correct orientation. The lanes as follows: (1) lambda DNA *Eco*R I/*Hin* dIII marker (Promega); (2) undigested pGVWR-gptNew; (3) pGVWR-gptNew digested with *Sal* I and (4) *Eco*R V; (5) undigested pGVWR-WG and pGVWR-WG digested with *Sal* I (6) and *Eco*R V (7).

7.3.2 Molecular cloning of a Mokola virus glycoprotein gene in a vaccinia virus transfer vector

The Mokola virus glycoprotein gene was subcloned from a recombinant mammalian expression vector, pBUDCE4.0-MokG in the *Pst* I site of the transfer vector, pGVWR-gptNew. The insertion of the gene was shown by digestion of clones with *Pst* I to yield DNA bands of 1 600 and 5 500 base pairs. The direction of the insertion of the gene was determined by digestion with double digestion with *Sac* I and *Eco*R V enzyme which yielded DNA bands of 1 700 and 5 400 base pairs for constructs with the gene in the correct orientation and 500 and 7600 base pairs for constructs with the gene in the wrong orientation.

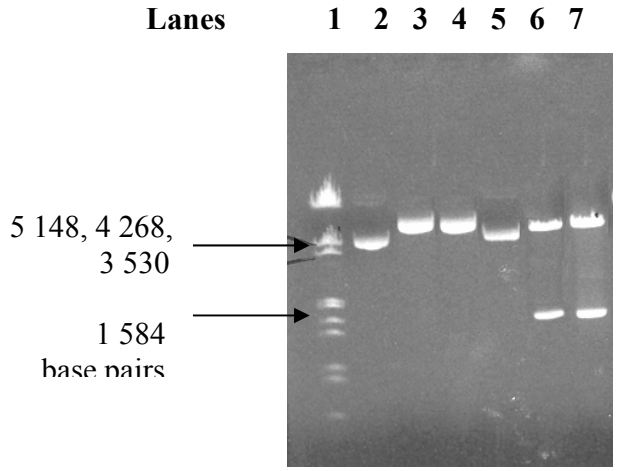


Figure 7.5: Molecular cloning of a Mokola virus glycoprotein gene in the vaccinia transfer vector, pGVWR-gptNew. In lanes (1) the lambda DNA marker *Eco*R I/*Hin* dIII (Promega); (2) undigested pGVWR-gptNew; (3) and (4) pGVWR-gptNew digested with *Pst* I, and *Sac* I/*Eco*R V, respectively. Undigested pGVWR-MG was run in lane 5. Lanes (6) and (7) represent pGVWR-MG digested with *Pst* I and *Sac* I/*Eco*R V respectively.

7.3.3 Generation of recombinant vaccinia viruses

Recombinant vaccinia viruses that carry either a rabies, Mokola or WCB virus glycoprotein were generated. Vaccinia virus Copenhagen was used as the parent virus for the generation of these single foreign antigen encoding recombinants. The rabies glycoprotein recombinant was already prepared but still harbored residual wild type virus background. The recombinant encoded a neomycin gene that affords resistance to the antibiotic G418. A pure recombinant was isolated after six rounds of plaque purification under G418 selection. The purity of the recombinant was analyzed by PCR of the HA gene region (figure 7.7). The primer pair, HA+ and HA-, annealed in the region flanking the hemagluttinin gene of the vaccinia virus genome to yield an amplicon of approximately 640 base pairs. With the recombinant virus the amplicon was about 4 000 base pairs. The Mokola and WBV glycoprotein encoding constructs were generated after transfection of the recombinant vaccinia virus transfer vectors in vaccinia virus infected cell culture. The homogenous recombinants were subsequently isolated after six rounds under MPA resistance selection. The purity of these recombinants was monitored by

PCR of the TK region of the parent genome (figure 7.6). PCR with the VacTKl and VacTKr primer pair yielded an amplicon of roughly 300 base pairs with the vaccinia virus genomic DNA as template. In the case of the recombinant viruses the band sized at 3 000 base pairs.

In addition, recombinant viruses that carry rabies and Mokola, rabies and WCB or two rabies virus glycoprotein genes were also constructed. A recombinant vaccinia virus encoding a rabies virus glycoprotein gene (VV-RG) was used as a parent virus for the construction of these double antigen encoding recombinants. And the recombinants were generated upon transfection of cell culture infected with VV-RG. The recombinants were isolated after six rounds under MPA selection. The homogeneity of the double constructs was analyzed by PCR TK region of the vaccinia virus genome (figure 7.6) and verified with PCR of the HA region (figure 7.7).

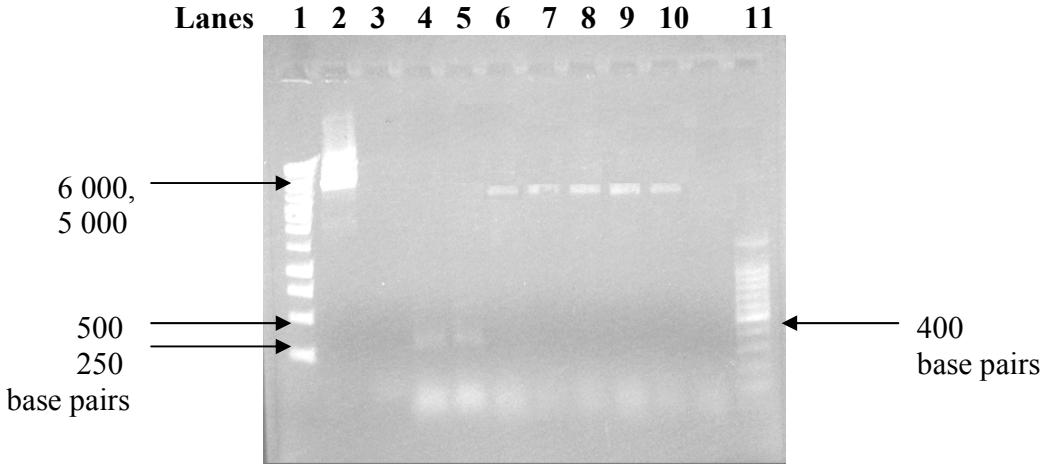


Figure 7.6: PCR amplicons obtained with the VacTKl and VacTKr primers that anneal in the regions flanking the TK region of the vaccinia virus Copenhagen genome. In lane (1) 1Kb ladder marker (Promega) and lane (11) the 100 bp marker (Promega). Templates used to yield these amplicons were (2) pGVWR-RabGEGFP plasmid for a positive control, (3) negative control, (4) vaccinia virus genomic DNA extract as positive control, (5) VV-RG, (6) VV-MG, (7) VV-WG, (8) VV-RGRG, (9) VV-RGMG and in lane (10) VV-RGWG.

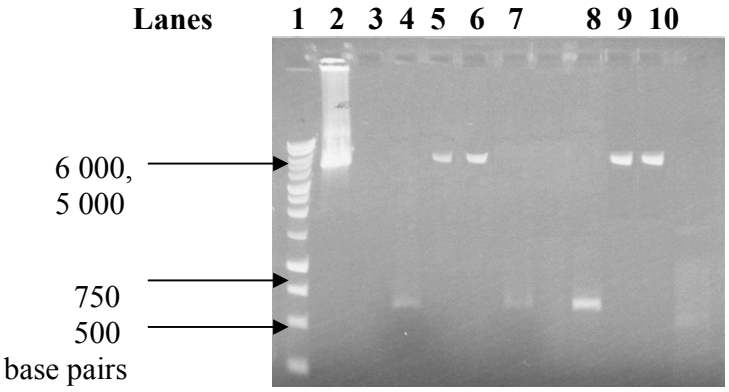


Figure 7.7: PCR of the HA region of parent and recombinant vaccinia viruses. A molecular weight marker was run in lane 1 (1 Kb ladder, Promega). The lanes represent the amplicons yielded with (2) pBRExpress-rabies glycoprotein plasmid positive control, (3) negative control, (4) vaccinia virus genomic DNA extract positive control, (5) VV-RG, (6) VV-RGRG, (7) VV-MG, (8) VV-RGMG, (9) VV-WG and (10) VV-RGWG.

7.3.4 Expression of lyssavirus glycoproteins from recombinant vaccinia viruses

The expression of the different glycoproteins from the recombinant viruses was confirmed by indirect immunofluoresence assay. The glycoproteins were probed with antibodies that bind specifically to the rabies, Mokola or WCBV glycoproteins. Expression of the glycoprotein genes was confirmed from all the constructs (figure 7.8 and 7.9). Background signals were very low for all three the antibodies used, whereas the positive controls delivered intense and specific signals (results not shown). No cross-reactivity was noted between the antibodies and heterologous glycoproteins, i.e. the Mokola glycoprotein monoclonal did not cross-react with the WCBV or rabies virus glycoproteins (results not shown). Although expression from both of the rabies virus glycoproteins from the VV-RGRG construct was not distinguished, expression of these particular genes was confirmed from the single gene constructs.

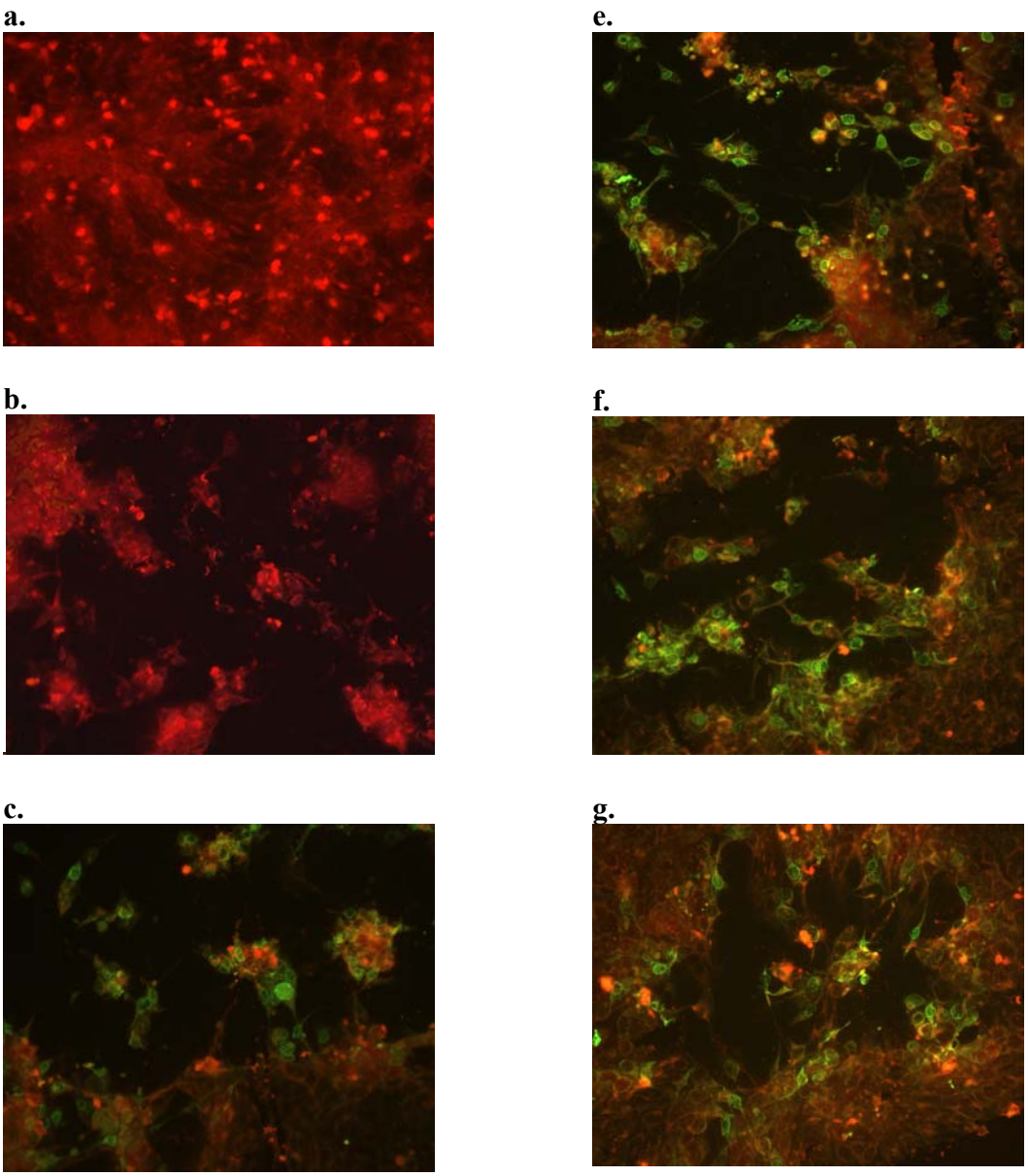


Figure 7.8: Representative images of the results of the indirect immunofluoresence assays with the anti-rabies virus glycoprotein monoclonal. Typical findings for the uninfected and vaccinia virus virus infected cell cultures are shown in (a) and (b). (c), (d), (e), (f) and (g) depict expression of the rabies virus glycoprotein from VV-RG, VV-RGRG, VV-RGMG and VV-RGWG respectively. The magnification of the images was 200 times (20X objective and 10X for the eyepiece of the microscope).

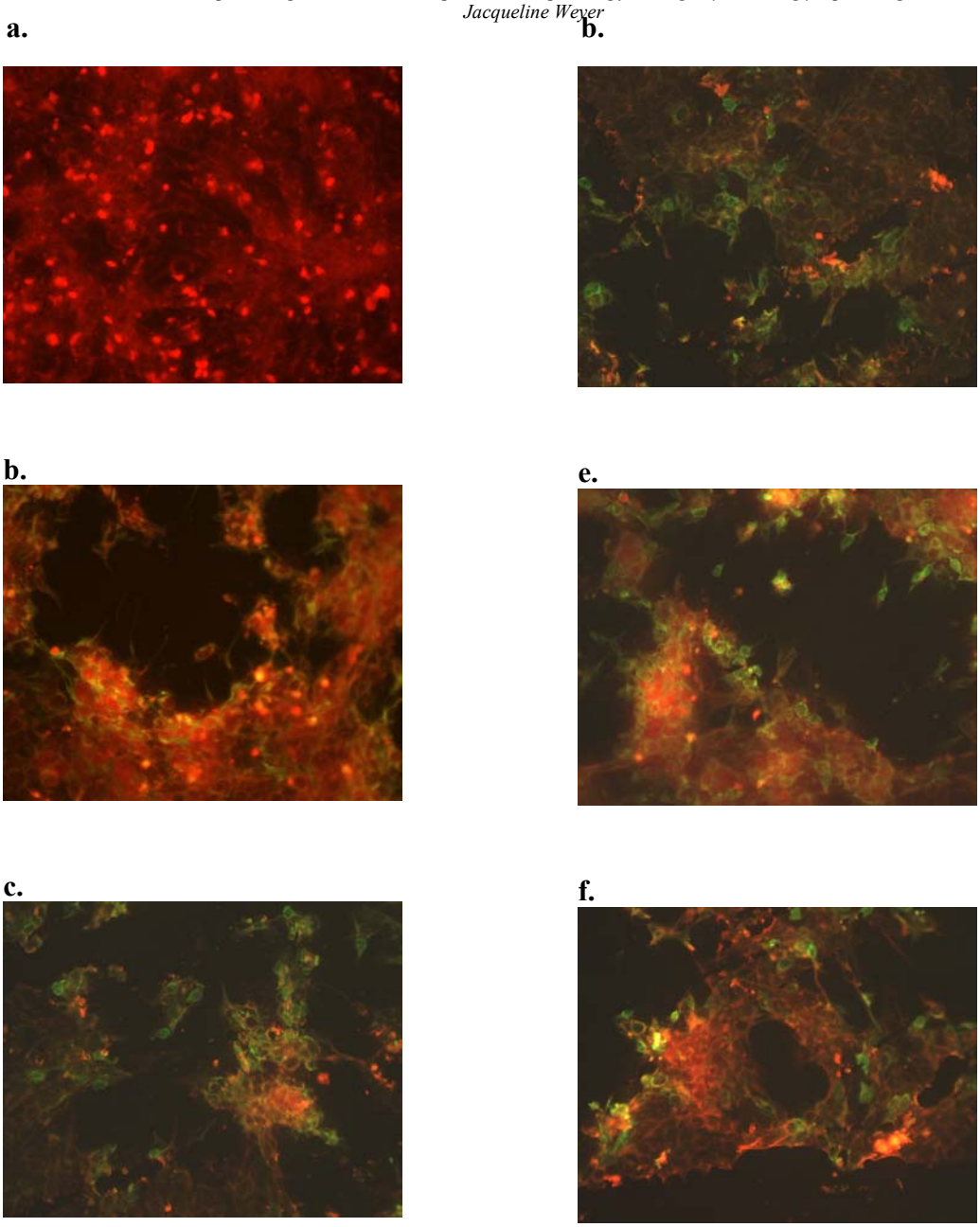


Figure 7.9: Indirect immunofluoresence with anti-mokola glycoprotein monoclonal and polyclonal anti-WCBV serum. In (a) uninfected Vero culture and (b) vaccinia virus infected Vero culture stained with anti-mokola monoclonal. (c) and (d) depicts expression of mokola glycoprotein from VV-MG and VV-RGMG. Whilst (e) and (f) depict the expression of WCBV glycoprotein from VV-WG and VV-RGWG using the anti-WGBV serum. Controls for the anti-WCBV serum is not shown but is comparable to (a) and (b).

7.3.5 Preparation of WCBV challenge material

Suckling mouse brain homogenates of WCBV were diluted in a 10-fold serial dilution and mice received these preparations intracranially. Mice were either killed by euthanasia when clinical signs appeared or collected upon death. The animals started showing symptoms by day 7 after inoculation and started dying or were killed by euthasia from day 8 through day 18. The symptoms observed included ruffled fur, seizures, general wasting and hemorrhaging from the eyes. The animals' brains were collected aseptically and tested for presence of rabies virus antigens with the dFA test (results not shown). The $MICLD_{50}$ per 30 µl of the preparation was calculated to be $10^{3.75}$ using the Reed and Muench approach (calculations not shown).

7.3.6 Survival studies and humoral responses in mice

Single antigen expressing vaccines (VV-RG, VV-MG and VV-WG) protected mice against lethal intracerebral challenge with homologous virus (figure 7.10 and tables 7.3-7.5). These constructs elicited measurable virus neutralizing responses by day 7 after primary immunization (tables 7.3-7.5). A five-fold increase in virus neutralizing antibody titer was measured on day 21, 7 days after a booster immunization, for animals immunized with VV-RG and VV-WG. A six-fold increase in titer was measured for the animals that received the VV-MG vaccine.

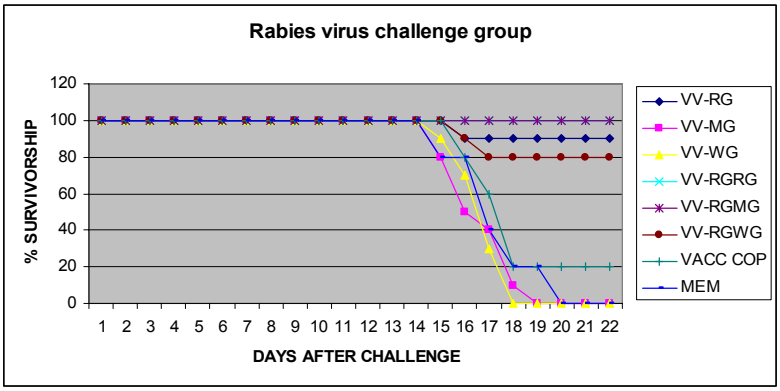
The double antigen expressing vaccines (VV-RGRG, VV-RGMG, and VV-RGWG) protected mice against lethal intracerebral challenge with homologous viruses (figure 7.8). The rabies virus neutralizing antibody responses increased three, five and seven-fold in animals after receiving the second dose of VV-RGRG, VV-RGWG and VV-RGMG.

Control animals vaccinated with either parent vaccinia virus or MEM succumbed from challenge with rabies, Mokola or WCB viruses respectively (figure 7.10 and tables 7.3-5).

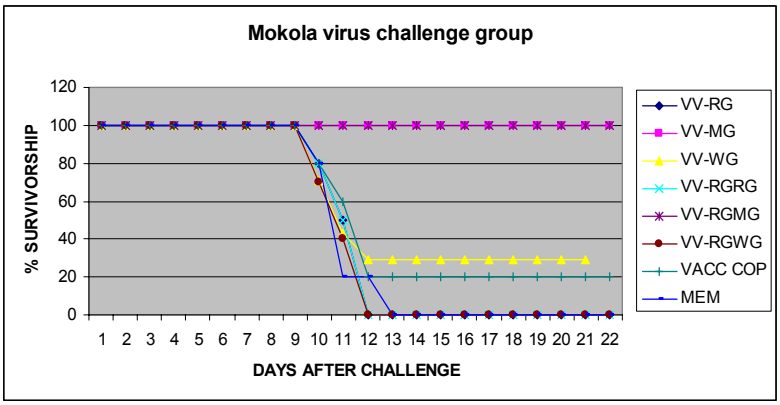
Statistically there was no significant difference in the levels of rabies virus neutralizing antibodies elicited by VV-RG and VV-RGRG. The levels of rabies virus neutralizing antibodies elicited after vaccination with VV-RG, and VV-RGMG and VV-

RGWG also did not differ significantly. The same was true for Mokola virus neutralizing antibody titers after vaccination with VV-MG and VV-RGMG, and WCBV virus neutralizing antibody titers in mice that received VV-WG or VV-RGWG.

a)



b)



c)

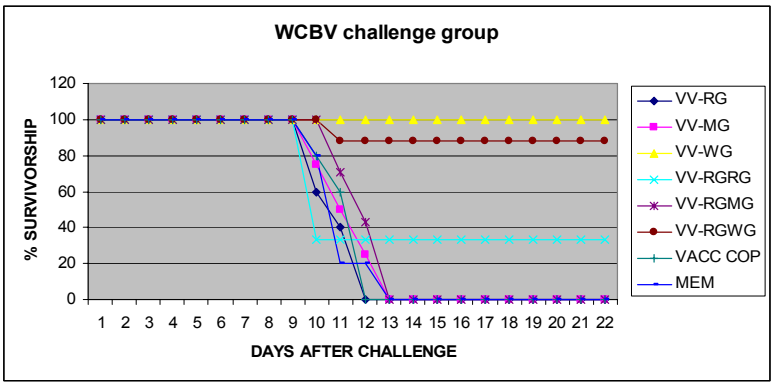


Figure 7.10: Survivorship of mice vaccinated with recombinant vaccinia virus vaccines (as indicated in the legend) after challenge with (a) street rabies virus, (b) Mokola virus or (c) WCBV.

Table 7.3: Pre-challenge rabies virus neutralizing antibody titers[a] and survivorship[b] of mice vaccinated with recombinant vaccinia viruses and challenged with street rabies virus. The titers are noted for day 0 (naïve sera), day 7 (seven days after primary vaccination) and day 21 (7 days after booster vaccinations).

GROUP	DAY 0[A]	DAY 7[B]	DAY 21[C]	% SURVIVORSHIP
VV-RG	< 1.00	**1.7 (1.39 - 2.01)**	**2.45 (1.58 - 2.81)**	90 (9/10)
VV-RGRG	< 1.00	**1.7 (1.08 - 2.01)**	**2.01 (1.38 -2.70)**	100 (10/10)
VV-MG	< 1.00	<1.00	<1.00	0 (0/10)
VV-RGMG	< 1.00	**1.63 (1.0 -1.9)**	**2.48 (1.90 –2.86)**	100 (10/10)
VV-WG	< 1.00	<1.00	<1.00	0 (0/10)
VV-RGWG	< 1.00	**1.7 (1.0 -1.9)**	**2.10 (1.4 – 2.7)**	80 (8/10)
Vacc Cop	< 1.00	< 1.00	< 1.00	20 (1/5)
MEM	< 1.00	< 1.00	< 1.00	0 (0/5)

[a] geometric mean titers (range of titers), log base 10, as determined with RFFIT
[b] number of animals surviving per group

Table 7.4: Pre-challenge Mokola virus neutralizing antibody titers[a] and survivorship[b] of mice vaccinated with recombinant vaccinia viruses and challenged with Mokola virus. The titers are noted for day 0 (naïve sera), day 7 (seven days after primary vaccination) and day 21 (7 days after booster vaccinations).

GROUP	DAY 0[A]	DAY 7[B]	DAY 21[C]	% SURVIVORSHIP
VV-RG	< 1.0	< 1.0	< 1.0	0 (0/10)
VV-RGRG	< 1.0	< 1.0	< 1.0	0 (0/10)
VV-MG	< 1.0	**2.18 (1.94 -2.52)**	**2.86 (2.71 – 3.01)**	100 (10/10)
VV-RGMG	< 1.0	**1.98 (1.15 – 2.43)**	**2.84 (2.18 - 3.08)**	100 (9/9)
VV-WG	< 1.0	< 1.0	**1.08 (1.0 -1.28)**	29 (2/7)
VV-RGWG	< 1.0	< 1.0	< 1.0	0(0/10)
Vacc Cop	< 1.0	< 1.0	< 1.0	0 (0/5)
MEM	< 1.0	< 1.0	< 1.0	0 (0/5)

[a]geometric mean titers (range of titers), log base 10, as determined with RFFIT
[b] number of animals surviving per group

Table 7.5: Pre-challenge WCBV neutralizing antibody titers[a] and survivorship[b] of mice vaccinated with recombinant vaccinia viruses and challenged with WCBV. The titers are noted for day 0 (naïve sera), day 7 (seven days after primary vaccination) and day 21 (7 days after booster vaccinations).

GROUP	DAY 0[A]	DAY 7[B]	DAY 21[C]	% SURVIVORSHIP
VV-RG	< 1.0	< 1.0	< 1.0	0 (0/10)
VV-RGRG	< 1.0	< 1.0	**1.13 (1.0 – 1.4)**	33 (1/3)
VV-MG	< 1.0	< 1.0	< 1.0	0 (0/8)
VV-RGMG	< 1.0	< 1.0	< 1.0	0 (0/7)
VV-WG	< 1.0	**1.7 (1.7)**	**2.48 (2.05 – 2.83)**	100 (7/7)
VV-RGWG	< 1.0	**1.7 (1.39 - 2.01)**	**2.10 (1.4 – 2.7)**	88 (7/8)
Vacc Cop	< 1.0	< 1.0	< 1.0	0 (0/5)
MEM	< 1.0	< 1.0	< 1.0	0 (0/5)

[a] geometric mean titers (range of titers), log base 10, as determined with RFFIT
[b] number of animals surviving per group

7.3.7 Cross-reactivity and cross-protection in mice

Animals that were immunized with VV-RG were not protected against challenge with Mokola or WCB viruses (figure 7.10). These results were mirrored in the lack of neutralization of rabies virus (CVS-11) with sera collected form these animals (table 7.3-7.5). Similar observations were made for mice that received VV-MG (figure 7.10 and table 7.3-7.5). The construct did not elicit protection against rabies or WCBV challenge. Once again these results were confirmed with the lack of neutralization of sera with rabies or WCB viruses.

33 % of mice vaccinated with VV-RGRG survived challenge with WCBV (table 7.5). Only three animals were tested in this group, since the rest of the group succumbed to the intracranial inoculation procedure. The sera of the surviving animal collected on day 7 after primary vaccination did not cross-neutralize WCBV, however, sera collected on day 21 did cross-neutralize WCBV. The titer of neutralizing antibodies was low, 1.13. The VV-RGRG construct did however not induce neutralizing responses against Mokola

virus and all the animals that received this vaccine and was challenged with Mokola virus succumbed.

The same observation was made for two of seven animals that received VV-RGWG as vaccine. These two animals survived challenge with Mokola virus. Once again no Mokola virus neutralizing antibodies could be measured from sera collected on day 7. Both animals had a low level of neutralizing immunoglobulin at 1.28 (table 7.4).

The VV-RGMG vaccine did not elicit any protective responses in mice against challenge with WCBV.

Sera were also tested against Lagos Bat virus for cross-reactivity. The sera collected from mice vaccinated VV-MG and VV-RGMG cross-neutralized Lagos Bat virus in RFFIT. Although the difference in cross-neutralizing titers from VV-MG and VV-RGMG vaccinated mice was not significant, the geometric mean titers elicited by VV-RGMG was lower on both day 7 and 21.

Table 7.6: Lagos Bat virus neutralizing antibody titers[a] of mice vaccinated with recombinant vaccinia viruses. The titers are noted for day 0 (naïve sera), day 7 (seven days after primary vaccination) and day 21 (7 days after booster vaccinations).

GROUP	DAY 0[A]	DAY 7[B]	DAY 21[C]	% SURVIVORSHIP
VV-RG	< 1.0	<1.0	<1.0	N/A
VV-RGRG	< 1.0	<1.0	<1.0	N/A
VV-MG	< 1.0	**1.49**	**2.36**	N/A
VV-RGMG	< 1.0	**1.15**	**2.07**	N/A
VV-WG	< 1.0	< 1.0	< 1.0	N/A
VV-RGWG	< 1.0	< 1.0	< 1.0	N/A
Vacc Cop	< 1.0	<1.0	<1.0	N/A
MEM	< 1.0	<1.0	<1.0	N/A

[a] geometric mean titers (range of titers), log base 10, as determined with RFFIT

7.4 Discussion

The public health burden from the non-rabies lyssaviruses that are not included in the protection spectrum of commercial vaccines remains largely obscure. The lack of epidemiological data on these viruses is primarily attributed to lack of active surveillance

in countries where these viruses have been isolated. Only a number of isolates of both Mokola and Lagos Bat virus have been collected. Lagos Bat virus is the only lyssavirus that has not yet been isolated from humans, and only two isolations of Mokola virus from humans have been reported (Familusi and Moore, 1972; Familusi *et al*., 1972). Only a single isolate of WCBV has been made (Botvinkin *et al*., 2003). Nevertheless, judging from the distribution of the postulated reservoirs of these viruses, the epizootiology of these viruses may be important for animal and human health (Hanlon *et al*., 2005). The isolation of Mokola virus from a diversity of hosts, including shrews (*Crocidura* spp), a dog, domestic cats and a rodent (*Lophuromys sikapusi*) indicates the virus's ability to cross the species barrier and underscores this latter statement (Nel *et al*., 2000).

In this study the cross-protective capacity of vaccinia virus vectored vaccines expressing different lyssavirus glycoprotein genes were tested. The vaccinia vaccine model has been extensively studied for the expression of rabies virus proteins and has been subsequently studied in various animal models, providing us with well studied model. More to the point, vaccinia viruses have been specifically proven to be effective carriers of rabies virus glycoproteins[37] (Kieny *et al*., 1984).

The latter fact has once again been underlined in this study. Recombinant vaccinia viruses expressing either the rabies, Mokola or WCBV glycoproteins have been proven efficacious in mice *via* the intramuscular route. Not only were substantial levels of specific virus neutralizing antibodies measured only a week after primary immunization, but with all but one of the constructs an anamnestic response of at least a five-fold increase in neutralizing antibody titers were measured a week after booster administrations. These vaccine constructs protected mice against lethal intracranial challenge with homologous virus. The same observations were made for the vaccines expressing two lyssavirus glycoproteins, either two rabies, rabies and Mokola or a rabies and WCBV glycoprotein genes. The double antigen vaccines elicited virus specific neutralizing responses that were comparable to that elicited by the single antigen expressing vaccines. There was also not a significant difference in the survivorship of animals that received the single or double antigen vaccines in this study.

[37] refer to sections Part One, Chapter Two, section 2.1.3.2 and 2.2.3

It has been shown by previous studies that recombinant rabies viruses expressing two glycoprotein genes concomitantly produced more glycoprotein and consequentially more neutralizing responses (Faber *et al*., 2002). It has also been suggested that increased virus neutralizing antibody titers (particularly with the Pittman Moore strain of rabies virus) are required for neutralization of the non-rabies lyssaviruses (Brookes *et al*., 2005b; Hanlon *et al*., 2005). Considering these statements it was anticipated that that a double rabies glycoprotein expressing vaccinia virus would induce not only more neutralizing responses, but possibly confer enhanced neutralization of the non-rabies lyssaviruses. In this study, it was however found that this vaccine elicited comparable titers of rabies neutralizing responses with the single glycoprotein expressing vaccine. Enhanced cross-reactivity of sera induced by the double rabies glycoprotein vaccine with Mokola, Lagos Bat and WCB virus was not observed. Notably one mouse that received the double rabies glycoprotein vaccine, survived the challenge with WCBV and also had low anti-WCBV antibody titer at day 21. Relatively high doses of vaccine were administered in this study, and it is possible that differences between the single and double antigen vaccines may only become apparent when tested at lower doses.

In accordance with previous reports, the rabies glycoprotein vaccine did not cross-protect against Mokola or against WCBV (Nel *et al*., 2003; Hanlon *et al*., 2005). Sera from animals that received the rabies glycoprotein vaccine did also not cross-react with Lagos Bat virus in RFFITs. The mortality rates of mice that were immunized with the rabies virus glycoprotein vaccine and then challenged with Mokola or WCB viruses were 100 %. These observations were substantiated by the lack of cross-reactive serological responses with these sera. Concomitantly the Mokola and WCB virus glycoprotein vaccines did also not cross-protect against rabies virus challenge. Once again these findings were supported by a lack of neutralizing responses as tested with the RFFIT. The Mokola glycoprotein vaccine did not offer any protection against challenge with WCBV, and so also for the WCB glycoprotein vaccine and Mokola virus challenge.

It can be eluded that a subunit vaccine that express Mokola and rabies glycoprotein should offer protection against most of the lyssaviruses and it appears that WCBV antigens should be incorporated in addition to recombinant vaccines.