Investigating the Antigenicity of the Lyssavirus Glycoprotein

A thesis submitted for the degree of Doctor of Philosophy

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Declaration

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree.

Parts of the introduction to this thesis have been published by the author:

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Summary

Rabies, the archetypal lyssavirus, is one of the most feared viruses known to man and globally, is the cause of more than 55,000 deaths per year. Alongside rabies virus, numerous related lyssaviruses exist that are also capable of causing fatal disease, clinically indistinguishable from rabies. Whilst the human burden of these non-rabies lyssaviruses remains unknown, fatalities have been reported.

The lyssavirus glycoprotein is the sole target for virus neutralising antibodies and several amino acid epitopes have been linked to virus neutralisation. Lyssaviruses are genetically and antigenically categorised into phylogroups that indicate the level of protection afforded by current vaccines. It is generally accepted that an antibody response to the currently available rabies vaccines affords protection against all viruses that are categorised into phylogroup I. However, this antibody response does not protect against lyssavirus species within phylogroups II and III. Indeed, experimental data has shown that the antibody repertoire induced by rabies virus vaccines is unable to neutralise viruses in these phylogroups. In this study we have generated lentivirus pseudotypes representing all currently defined lyssaviruses as well as including chimeric lyssavirus glycoproteins that have had their antigenic sites swapped between phylogroup I and II viruses. Using the wildtype lyssavirus pseudotypes we have confirmed a strong level of intra-phylogroup neutralisation in addition to very limited inter-phylogroup neutralisation. The antigenic site swap constructs have shown an alteration in both glycoprotein functionality alongside altered neutralisation profiles using a variety of vaccine induced and divergent lyssavirus specific sera.

Promising cross protective candidates have been cloned into a vaccine strain full length backbone and reverse genetics has enabled rescue of these viruses. The growth kinetics of recombinant viruses have been studied *in vitro* and an investigation into the pathogenicity and degree of vaccine induced protection against these viruses *in vivo* has been conducted.

Abbreviations Used in the Text

1X	One times concentration
3’	3 prime
5’	5 prime
°C	Degrees Celsius
%	Percent (where 1 ml in 100 ml is 1%)
A	Adenine
aa	Amino acid
ABLV	Australian bat lyssavirus
APHA	Animal and Plant Health Agency
APS	Ammonium persulphate
ARAV	Aravan virus
ASU	Animal Services Unit
BBLV	Bokeloh bat lyssavirus
BHK	Baby hamster kidney (cells)
bp	Nucleotide base pair
BSA	Bovine serum albumin
C	Cytosine
cDNA	Complementary DNA
CO_2	Carbon dioxide
CVS	Challenge virus strain
cm	Centimetre (10^{-2} m)
DC	Dendritic cell
DI	Defective interfering
DMEM	Dulbecco's Modified Eagle Medium

DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside-5'-triphosphate
DPBS	Dulbecco's PBS
dpi	Days post infection
ds	Double stranded
DUVV	Duvenhage virus
EBLV	European bat lyssavirus
EBLV-1	European bat lyssavirus type 1
EBLV-2	European bat lyssavirus type 2
EDTA	Ethylenediaminotetra-acetic acid
ELISA	Enzyme linked Immunosorbent assay
EM	Electron microscopy
ERIG	Equine rabies immune globulin
FAVN	Fluorescent antibody virus neutralisation test
FBS	Foetal bovine serum
Ffu/ml	Focus forming units per millilitre
FITC	Fluorescein Isothiocynate
For	Forward (in context of primers)
FP-T7	Fowlpox recombinant expressing T7 polymerase
G	Guanine
G	Glycoprotein
g	Gram
g / l	Grams per litre
x *g*	Centrifugation force
GFP	Green fluorescent protein

GMEM	Glasgow Modified Eagle Medium
h	Hour
HCl	Hydrogen chloride
HDCV	Human diploid cell vaccine
HEK 293T/17	Human embryonic kidney cell clone 17 expressing SV40 large T antigen
HIV	Human immunodeficiency virus
hpi	Hours post infection
HPLC	High-performance liquid chromatography
HRIG	Human rabies immune globulin
H_2O	Water
i.c.	Intra-cranial
i.m.	Intra-muscular
i.p.	intra-peritoneal
IF	Immunofluorescence
IFN	Interferon
IKOV	Ikoma lyssavirus
IRKV	Irkut virus
ISRE	IFN stimulated response element
IU	International unit
kb	Kilobase pair (10^{-3} bp)
kDa	Kilodalton
KHUV	Khujand virus
kg	Kilogram(10^3 g)
L	Large (polymerase)
l	Litre

LB	Luria Bertaini
LBV	Lagos bat virus
LBV A	Lagos bat virus lineage A
LBV B	Lagos bat virus lineage B
LBV C	Lagos bat virus lineage C
LBV D	Lagos bat virus lineage D
LLEBV	Lleida bat lyssavirus
LMP	Low melting point
M	Matrix
M	Molar
m	Metre
mAb	Monoclonal antibody
Maxi	Maximum
MDA-5	Melanoma-associated gene 5
mFAVN	Modified fluorescent antibody virus neutralisation test
mg	Milligram (10^{-3} g)
min	Minute
ml	Millilitre
mM	Millimolar (10^{-3} M)
mm	Millimetre (10^{-3} m)
mmol	Millimole (10^{-3} mol)
MOI	Multiplicity of infection
MOKV	Mokola virus
MP	Milwaukee protocol
mRNA	messenger RNA

N	Nucleocapsid
NaCl	Sodium chloride
NaOH	Sodium hydroxide
ng	Nanogram (10^{-9} g)
ng / µl	Nanograms per microlitre
nm	Nanometre (10^{-9} m)
nmol	Nanomole (10^{-9} mol)
NGS	Next generation sequencing
nns	Negative sense single stranded
nt	Nucleotide
OIE	Office International des Epizooties
ORF	Open reading frame
P	Phosphoprotein
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
pg	Picogram (10^{-12} g)
pmol	Picomole (10^{-12} mol)
PNA	Pseudotype neutralisation assay
P/S	Penicillin/Streptomycin
PT	Pseudotype virus
RABV	Classical rabies virus
RdRp	RNA-dependent RNA polymerase
Rev	Reverse

RHIS	Rabbit hyperimmune serum
RIG	Rabies immune globulin
RIG-I	Retinoic acid-inducible gene I
RLR	RIG-I like receptor
RNA	Ribonucleic acid
RNase	Ribonuclease
RNP	Ribonucleoprotein
rpm	Revolutions per minute
RT	Reverse transcriptase
rt	Room temperature
RT-PCR	Reverse-transcription PCR
s	Second
s.c.	Sub-cutaneous
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SHIBV	Shimoni bat virus
SOC	Tryptone/yeast extract bacterial media
ss	Single stranded
SSIII	Superscript III reverse transcriptase
SV40	Simian virus 40
T	Thymine
TAE	Tris-acetate EDTA
$TCID_{50}$	50% Tissue culture infectious dose
TE	Tris-EDTA.
TEMED	N,N,N’,N’-tetramethylethenediamine

TENS	Tris/EDTA/NaOH/SDS buffer
TLR	Toll-like receptor
TM	Transmembrane
T_m	Melting temperature
Tris	Tris [hydroxymethyl] aminomethane
TBS	Tris buffered saline
U	Uracil
μg	Microgram (10^{-6} g)
μg / μl	Micrograms per microlitre
μl	Microlitre (10^{-6} l)
μM	micromolar (10^{-6} M)
μmol	Micromole (10^{-6} mol)
UTR	Untranslated region
V	Volts
VLP	Virus-like particles
VSV	Vesicular stomatitis virus
WCBV	West Caucasian bat virus
WHO	World Health Organisation
wt	Wild type

Chapter 1: Introduction

1.1 Lyssaviruses

The lyssaviruses constitute an important group of viruses that are of significance to both human and animal health. All viruses within this genus are highly neurotropic and are capable of causing a fatal encephalitis, known as rabies. The prototypic lyssavirus is rabies virus (RABV) which is primarily transmitted through the bite of an infected dog and causes a higher burden of disease in both humans and animals than any other lyssavirus. Indeed, the term 'lyssa' is of Greek origin and is believed to refer to a 'raging dog' (Neville, 2004). Of the remaining lyssaviruses, all have been detected in bats with only two exceptions, Mokola virus and Ikoma lyssavirus. Despite safe and effective prophylactic and post-exposure tools being available RABV remains endemic across much of Africa and the Indian subcontinent. Members of the lyssavirus genus are able to infect and cause disease in a very wide host range, with theoretically all mammalian species being susceptible to infection (Rupprecht *et al.*, 2008). Susceptibility to infection with these viruses does vary with several factors being hypothesised to contribute to the duration of the incubation period, progression of disease and overall outcome of infection including: site of wound, virus dose, infecting strain and immunocompetence of the host. Despite the availability of tools to control rabies infection, the presence of virus in sylvatic populations has made elimination in certain mammalian species difficult and eradication problematic. From a human perspective, a lack of awareness of the disease and variation in incubation period following infection can mean that appropriate prophylactic tools are not sought and fatalities occur as where clinical disease develops, infection with lyssaviruses is invariably fatal. In 2010 the WHO estimated that between 26,400 and 61,000 people died from rabies, with approximately 33,000 deaths in Asia, 26,000 deaths in Africa and less than 100 deaths in Latin

America, North America and Europe combined (WHO, 2005; 2013).

1.1.1 Classification

Lyssaviruses are classified within the Family Rhabdoviridae, Order *Mononegavirales*. This Order contains three further families: the Bornaviridae, the Filoviridae and the Paramyxoviridae. All viruses within this Order contain linear, non-segmented, negative-sense, single-stranded ribonucleic acid (RNA) genomes. As such, for replication to occur, first viral mRNAs are transcribed, and following the accumulation of viral proteins within an infected cell, the antigenome sense RNA is generated as a full length positive sense replicative intermediate which acts as a template for replication to produce the negative sense genome (Whelan *et al.*, 2004). Transcription and replication tend to occur in the cytoplasm of infected cells for most members of the *Mononegavirales* with the exception of bornaviruses, nyavirus and the nucleorhabdoviruses which carry out transcription and replication in the host cell nucleus (Easton & Pringle, 2012). The majority of viruses classified within the *Mononegavirales* encode 5-10 genes in their genomes, although some have evolved to generate more through maximisation of coding potential (Pringle & Easton, 1997). Genetic reassortment is poorly characterised for most members of the *Mononegavirales*, most likely because the RNA genome is almost always very tightly encapsidated thus limiting the potential for hybridisation of an alternative template (Mebatsion *et al.*, 1996b). So far there have been two documented cases of genetic reassortment in *Mononegavirales*; one between two deletion mutants of Respiratory Syncytial Virus (RSV) which were able to produce just one reassortant virus despite being under optimal laboratory controlled conditions for reassortment (Spann *et al.*, 2003) and another case which detected two potential recombination events in a panel of 44 rabies viruses, both

within the polymerase protein (Liu *et al.*, 2011).

Within the *Mononegavirales*, different members of the Rhabdoviridae are able to infect a wide range of hosts including mammals, invertebrates, plants and fish, although to date only lyssaviruses and vesiculoviruses have been reported as the cause of clinical disease in animals and humans (Table 1.1). Alongside this, novel rhabdoviruses continue to be reported with Oita Rhabdovirus being isolated from a horseshoe bat being able to cause fatal encephalitis in mice up to 3 weeks of age (Iwasaki *et al.*, 2004), a human pathogen, Bas Congo virus, was found to cause a fatal haemorrhagic disease in humans (Grard *et al.*, 2012) and Fikirini virus which was isolated from a *Hipposideros vittatus* bat in Kenya (Kading *et al.*, 2013) being recently described.

1.1.1.1 Lyssavirus Phylogroup Classification

Within the lyssavirus genus, viruses are classified according to both genetic and antigenic data. Recently, there has been a shift in nomenclature from the definition of lyssavirus genotypes to that of lyssavirus species (ICTV, 2012). In addition to the separation of genetically distinct viruses into species, these viruses can also be divided into phylogroups using antigenic data (Horton *et al.*, 2010; ICTV, 2012; Nadin-Davis, 2007) (Figure 1.1).

This differentiation is mainly based on the degree of cross protection afforded by the current rabies vaccines between each of the viruses characterised. Genetic data allow reliable quantitative comparison of lyssavirus divergence (Marston *et al.*, 2007) and sequence data are available for at least one isolate from all 14 virus species classified within the lyssavirus genus. Across the open reading frame (ORF) encoding the G

Table 1.1: Rhabdovirus taxonomy, species, hosts and vector species. Adapted from (Healy *et al.*, 2013a)

Genus	Number of species	Species	Primary hosts	Vector
Ephemerovirus	5	Bovine ephemeral fever virus Adelaide river virus Berrimah virus Kotonkan virus Obodhiang virus	Cattle	Arthropods
Lyssavirus	14	Rabies virus (RABV) Duvenhage virus (DUVV) European bat lyssavirus type 1 (EBLV-1) European bat lyssavirus type-2 (EBLV-2) Mokola virus (MOKV) Lagos bat virus (LBV) Irkut virus (IRKV) Khujand virus (KHUV) Shimoni virus (SHIBV) West Caucasian bat virus (WCBV) Aravan virus (ARAV) Australian bat lyssavirus (ABLV) Bokeloh bat lyssavirus (BBLV) Ikoma virus (IKOV)	Dogs Humans Bats	N/A
Vesiculovirus	10	Vesicular stomatitis Alagoas virus Vesicular stomatitis Indiana virus Vesicular stomatitis New Jersey virus Chandipura virus Isfahan virus Carajas virus Cocal virus Maraba virus Piry virus	Cattle Arthropods Fish	Arthropods/ Annelids
Cytorhabdovirus	9	Lettuce necrotic yellows virus Northern cereal mosaic	Plants Aphids	Arthropods

		virus Strawberry crinkle virus Barley yellow striate mosaic virus Broccoli necrotic yellow virus Festuca leaf streak virus Lettuce yellow mottle virus Sonchus virus Wheat American striate mosaic virus		
Nucleorhabdovirus	11	Potato yellow dwarf virus Maize mosaic virus Sowthistle yellow vein virus Datura yellow vein virus Eggplant mottled dwarf virus Maize fine streak virus Maize Iranian mosaic virus Rice yellow stunt virus Sonchus yellow net virus Sowthistle yellow vein virus Taro vein chlorosis virus	Plants	Arthropods
Novirhabdovirus	4	Infectious haematopoietic necrosis virus Viral haemorrhagic septicaemia virus Snakehead virus Hirame rhabdovirus	Fish	Link to fish parasites
Sigmavirus	7	Drosophila melanogaster sigmavirus Drosophila affinis Sigmavirus Drosophila ananassae Sigmavirus Drosophila immigrans Sigmavirus Drosophila obscura Sigmavirus Drosophila tristis Sigmavirus Drosophila stabulans sigmavirus	Fruit flies	Arthropods
Perhabdovirus	3	Perch rhabdovirus Sea trout rhabdovirus	Fish	Unknown

		Anguillid rhabdovirus		
Tibrovirus	2	Tibrogargan virus Coastal Plains virus	Ticks Cattle	Arthropods
Tupavirus	2	Durham virus Tupaia virus	Birds	Unknown
Unassigned	4	Flanders virus Moussa virus Ngaingan virus Wongabel virus	Birds Mosquito Macropod/Midges	Unknown

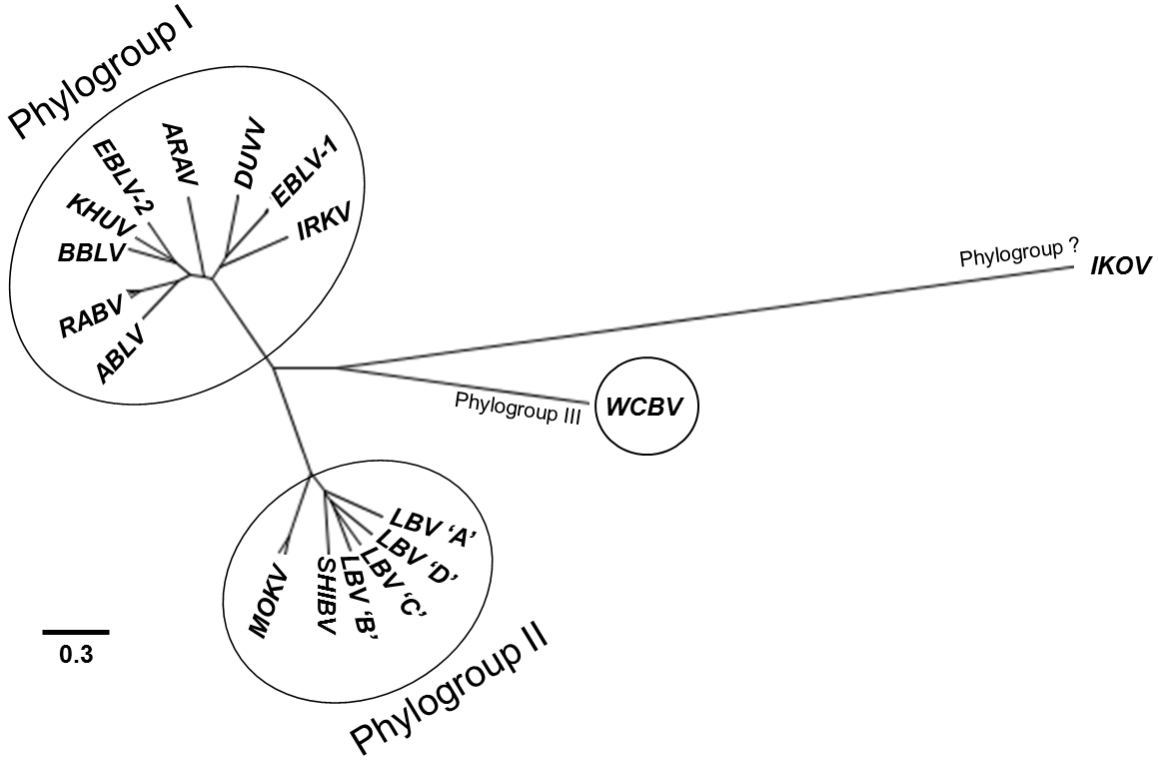


Figure 1.1: Bayesian phylogenetic analysis of glycoprotein ectodomain sequences. Representative lyssavirus species glycoproteins were aligned using BEAST (v1.4.8). The GTR+I model of nucleotide substitution and a chain length of 5 million were used. The maximum clade credibility tree was chosen using Tree annotator (v1.4.8) and viewed using Figtree (v1.1.2) estimated samples sizes were >100 and posterior probabilities at key nodes are shown. Scale bar represents substitutions per site. Accession numbers for each of the lyssavirus species analysed in this study are: ABLV AF801020, RABV CVS-11 EU352767, RABV Pitman Moore AJ871962, RABV Flury-HEP GU565704, BBLV JF311903, KHUV EF614261, EBLV-2 EF 157977, ARAV EF614259, DUVV EU293119, EBLV-1 EU293109 and EU293120, IRKV EF614260, MOKV HM623780 and EU293118, SHIBV GU170201, LBV 'B' EF547431, LBV 'C' EF547425, LBV 'D' GU170202, LBV 'A' EF547432, WCBV EF614258 and IKOV JN800509. Taken from (Evans *et al.*, 2012).

protein the lyssaviruses are at most 50% divergent at the nucleotide level while sharing only 46% identity at the amino acid level, as seen in Table 1.2.

There is a low degree of cross neutralisation between phylogroups I and II and also between both phylogroups and WBCV (Horton *et al.*, 2010). *In vivo* vaccine challenge experiments have shown reduced or no efficacy of current licensed rabies vaccines against viruses in phylogroup II (MOKV, LBV, SHIBV) (Badrane *et al.*, 2001) and phylogroup III (WCBV) (Hanlon *et al.*, 2005; Horton *et al.*, 2014). There is also evidence for variable vaccine efficacy against some viruses in phylogroup I (Brookes *et al.*, 2006; Brookes *et al.*, 2005; Fekadu *et al.*, 1988; Hanlon *et al.*, 2005) which suggests a gradual loss of vaccine protection corresponding to antigenic distance from vaccine strains.

1.1.2 Lyssavirus Genome Organisation and Diversity

All lyssaviruses are enveloped, deriving their envelope from the plasma membrane of the infected host cell upon budding; they exhibit the classic rhabdoviral bullet-like morphology. They generally measure ~75nm by 180nm (Warrell & Warrell, 2004). The genome organisation of members of the lyssavirus genus is relatively simple, consisting of between 11,000 and 12,000 nucleotides which encode just five genes: N, P, M, G and L (Dietzschold *et al.*, 2008). The genes all have a very conserved sequence across the order. The nucleoprotein (N) tends to be at the 3' end followed by the phosphoprotein (P) and matrix protein (M), this is followed by the glycoprotein (G) and the large polymerase (L) is always at the 5' end. This conserved order of genes may be an adaptation to enable control of protein expression (Hodges *et al.*, 2012). The genome arrangements for a representative of each of the genera within the Rhabdoviridae are

Nucleotide (% identity)	Amino acid (% identity)																	
	ABLV	IKOV	WCBV	LBV 'A'	LBV 'D'	LBV 'B'	LBV 'C'	SHIBV	MOKV	RABV CVS	RABV RV61	DUVV	EBLV 1	IRKV	ARAV	BBLV	EBLV 2	KHUV
ABLV		48	52	58	56	58	58	60	58	73	75	71	73	72	76	76	76	77
IKOV	52		48	50	47	47	48	49	48	47	48	46	48	48	46	47	48	47
WCBV	55	55		53	52	54	52	53	53	50	50	50	50	51	51	52	51	51
LBV 'A'	59	53	56		81	80	82	78	77	57	57	57	58	59	56	57	56	57
LBV 'D'	59	52	58	73		79	81	77	74	55	56	57	59	58	57	57	58	57
LBV 'B'	59	53	57	73	73		85	79	73	57	58	56	57	59	58	56	57	57
LBV 'C'	58	54	58	72	74	77		80	74	56	58	57	58	59	58	57	57	57
SHIBV	60	55	58	71	71	72	72		72	59	59	58	59	58	58	59	57	59
MOKV	57	52	57	69	71	69	67	67		58	58	57	58	58	57	57	57	57
RABV CVS	70	53	56	58	59	58	59	60	58		90	69	70	68	73	75	73	75
RABV RV61	71	53	54	59	59	60	60	61	59	87		71	73	69	75	77	76	78
DUVV	67	52	58	58	60	58	58	59	59	65	65		80	75	78	78	79	77
EBLV 1	68	53	57	60	62	59	60	62	61	66	68	73		80	84	78	80	79
IRKV	66	53	57	59	60	60	61	60	60	66	67	70	74		78	75	76	76
ARAV	70	53	57	58	61	60	60	61	60	70	69	70	74	70		83	85	83
BBLV	71	54	58	58	60	60	60	60	60	70	70	71	72	70	74		87	86
EBLV 2	71	53	57	58	61	61	61	60	60	70	71	72	74	70	76	77		88
KHUV	73	53	57	58	60	60	59	59	60	69	70	71	72	75	75	76	79	

Table 1.2: Nucleotide and amino acid identity of the lyssavirus G protein. Percentage identities are shown. Where identity is less than or equal to 60% they are shaded green. Viruses that have been tentatively classified into phylogroups are shaded accordingly. Taken from (Evans *et al.*, 2012)

displayed in Figure 1.2.

1.1.2.1 The Nucleoprotein

The nucleoprotein (N) is the most well conserved of the lyssavirus proteins (Mannen *et al.*, 1991) so is the main area studied in the determination of evolutionary relationships between lyssavirus isolates (Sacramento *et al.*, 1991). It is also the main target of diagnostic, epidemiological and immunological studies (Bourhy *et al.*, 1993; Kissi *et al.*, 1995).

The protein itself is approximately 450 amino acids in length and one of its main functions is the encapsidation of the viral genome. The core of the virus genome; the ribonucleoprotein complex (RNP) consists of the genomic RNA tightly encapsidated by N in association with the phosphoprotein and large polymerase (Tordo, 1996) and is the minimal essential replication unit (Schnell *et al.*, 2010).

N also plays a role in the regulation of transcription and replication (Kissi *et al.*, 1995). Accumulation of N in infected cells is thought to trigger a switch from transcription of the monocistronic messenger RNAs (mRNA) to transcription of the genome-length positive sense template RNA. It is this switch which induces replication of the negative sense RNA genome (Tordo *et al.*, 1988).

The N protein has also been implicated in the induction of B cell proliferation and T helper cell (T_h) responses, both of which can induce long lasting humoral immunity to RABVs (Koser *et al.*, 2004).

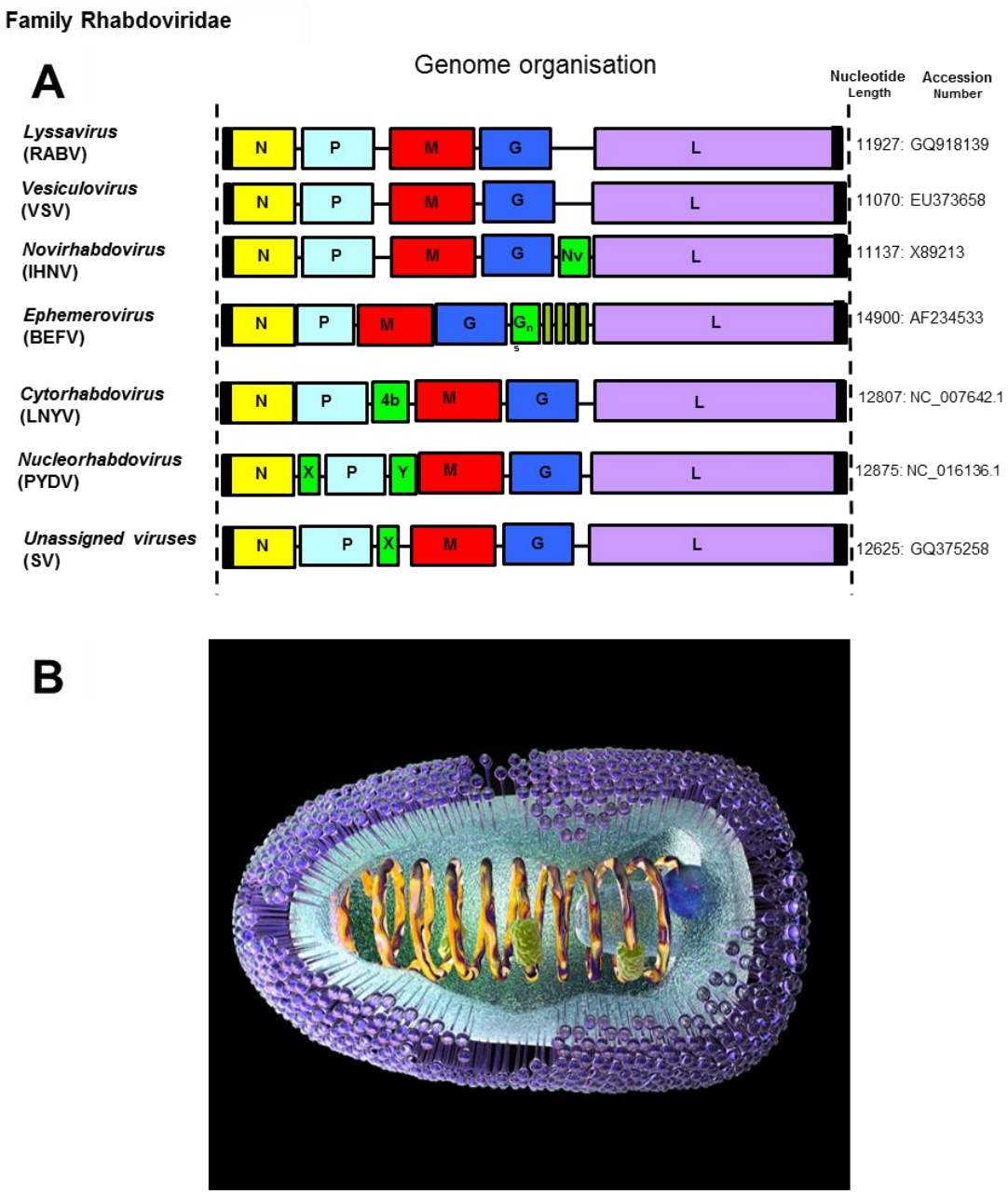


Figure 1.2: Genome organisation and virion morphology within the family Rhabdoviridae. (A) A schematic depicting the genome organisation for each genus within the family Rhabdoviridae. Gene open reading frame (ORF) sizes are not to scale and the full genome lengths have been standardised. Genome lengths and examples of a virus for each genus have been given. Genes additional to the minimal 5 are highlighted in green. (B) Schematic depiction of a rhabdoviral virion. The RNP core is shown in yellow with the P (green) and L (blue) surrounded by the matrix (pale blue) which is interspersed with numerous glycoproteins (purple) Adapted from (Healy *et al.*, 2013a).

1.1.2.2 The Phosphoprotein

The phosphoprotein (P) gene encodes a protein of between 297 and 303 amino acids (Tordo, 1996). It exists in two forms with a molecular weight of 38-41 kDa, depending on the degree of phosphorylation (Gupta *et al.*, 2000). It has been reported to play a role in viral transcription and also pathogenicity (Tan *et al.*, 2007). P is an essential component for the function of the large polymerase (L) protein (Marston *et al.*, 2007). It is also able to initiate interaction with the cytoplasmic dynein light chain LC8 which may contribute to the axonal transport of RABV in infected neurones (Lo *et al.*, 2001) and it may also play a role in down regulation of innate host immune responses post infection (Brzozka *et al.*, 2006).

1.1.2.3. The Matrix protein

The matrix (M) is a small protein; 23-24 kDa which plays multiple roles within the lyssavirus replication cycle. It is the main structural component of the virus, being proposed to form a layer between the glycoprotein and the RNP core which holds the virion in its distinctive bullet-shaped form (Graham *et al.*, 2008). It does this by interacting with the cytoplasmic tail of the glycoprotein at the cell membrane and promotes virus assembly and budding (Mebatsion *et al.*, 1999). It has also been suggested that M may play a role in regulation of the balance between transcription and replication of the viral genome (Finke *et al.*, 2003). Like P, it is thought M may be able to down regulate some host gene expression (Komarova *et al.*, 2007) and may also be involved in the induction of apoptosis of infected cells (Kassis *et al.*, 2004; Morimoto *et al.*, 1999).

1.1.2.4. The Glycoprotein

The glycoprotein (G) is composed of 512 to 524 amino acids (Marston *et al.*, 2007) and 65kDa in weight (Tordo, 1996). It is responsible for numerous functions as it is the only viral protein present on the virion surface. For this reason it plays an essential role in controlling virus and cell interactions (Rupprecht *et al.*, 2002) including: determination of cell tropism (Wunner *et al.*, 1984), fusion of the viral membrane and host cell endosome to release the nucleocapsid into the host cell cytoplasm (Gaudin *et al.*, 1993) and formation of the virion envelope by its interaction with M (Mebatsion *et al.*, 1999). The variation in the sequence of G between lyssavirus isolates is thought to play a role in the differing degrees of pathogenicity seen between different RABV strains or lyssavirus species (Faber *et al.*, 2005; Morimoto *et al.*, 2000; Yan *et al.*, 2002).

The G protein is the main immunogenic component of the virion, thus the primary target for virus neutralising antibodies (VNAs) (Benmansour *et al.*, 1991; Dietzschold *et al.*, 1982). G is also able to stimulate cytotoxic T cells (T_c) (Macfarlan *et al.*, 1986) and in combination with the induction of VNAs is the major protein responsible for the induction of protective immunity against challenge (Wiktor, 1985).

1.1.2.4.1 Antigenic Sites on the Glycoprotein

Present on the surface of the virion, the lyssavirus G protein is the sole viral component responsible for induction of a host antibody response and as such is the target of host neutralising antibodies (Gaudin *et al.*, 1992). The regions of the G protein responsible for the differences in antigenicity seen are ill defined. The G protein is relatively conserved in length across the genus with an ORF encoding between 512 and 534 amino acids. Furthermore all lyssavirus proteins contain 14 highly conserved cysteine residues

alongside the antigenic domains that contribute to the structure of the protein (Figure 1.3).

Currently, defined antigenic domains within the G protein include four major antigenic sites and one minor antigenic site. Historically, these have been defined through mapping of monoclonal antibody (mAb) binding sites as shown in Table 1.3.

The positions and a consensus amino acid sequence for each antigenic site are detailed in Table 1.4.

Antigenic site I is a region containing both conformational and linear epitopes as it is delineated by the monoclonal antibody mAb 509-6 that recognises a conformational epitope as well as antibody CR57 that recognises a linear epitope (Benmansour *et al.*, 1991). This site also appears to be the region recognised by the potent neutralising antibody, 62-7-13, a candidate antibody that has been proposed for inclusion in a monoclonal antibody based passive immunisation cocktail (Both *et al.*, 2013; Muller *et al.*, 2009).

Antigenic site II has two domains, IIa and IIb, and is thought to be present as a discontinuous conformational epitope (Benmansour *et al.*, 1991). Antigenic site II is proposed to be recognised by the neutralising antibody, E559 (Muller *et al.*, 2009).

Antigenic site III is continuous, though no site specific mAbs are able to bind unfolded protein which indicates that this epitope forms part of a loop on the protein surface and

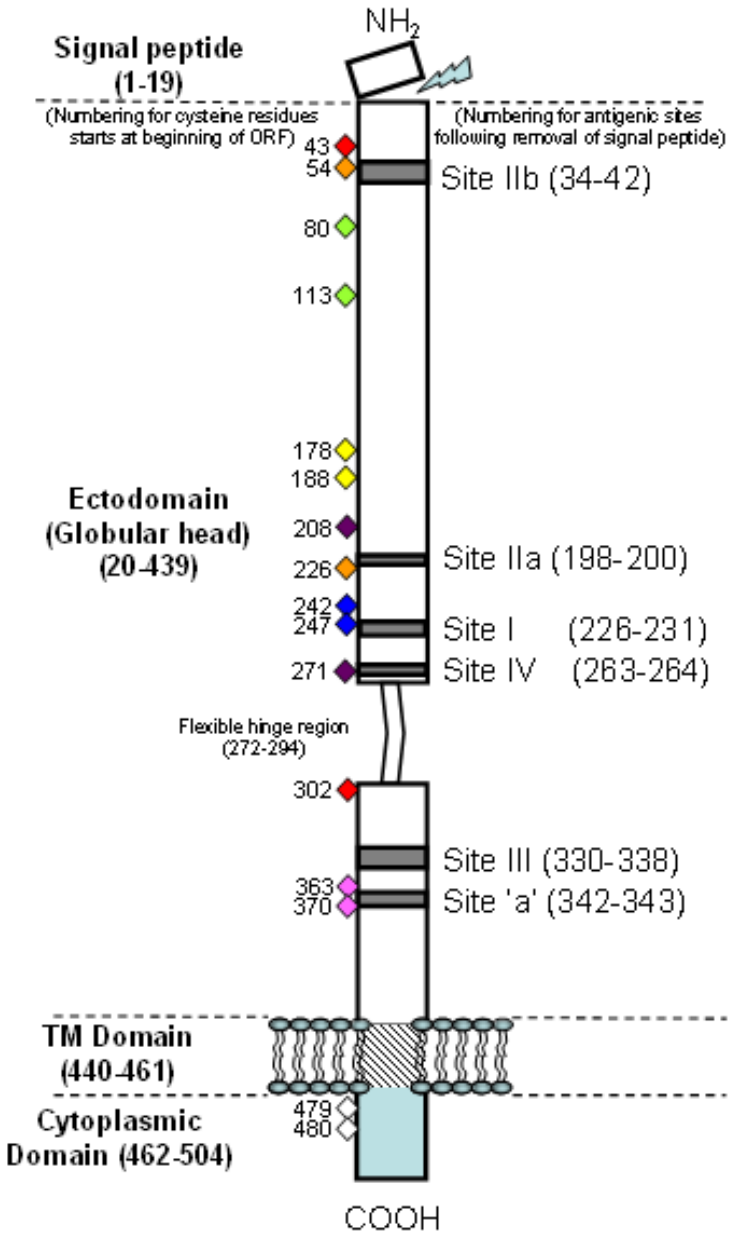


Figure 1.3: Schematic representation of the lyssavirus G protein. Defined antigenic sites and conserved cysteine residues are shown. C residues are coloured according to proposed linkages within the mature protein. TM: transmembrane; NH2: amino terminus; COOH: Carboxyl-terminus. Taken from (Evans *et al.*, 2012).

Table 1.3: Methods by which each of the proposed lyssavirus antigenic sites were identified. Amino acid position numbering is given following the removal of the signal peptide. The reference describing each specific mAb is given in superscript.

Antigenic Site	Position	Identification	Specific mAbs		Viral Source	Reference
I	226-231	mAb mapping	509-6[a,b]		CVS-11	**(Lafon *et al.*, 1983)**[a] **(Benmansour *et al.*, 1991)**[b]
II	IIb – 34-42 IIa – 198-200	mAb mapping	231-11[a] 220-8[a] 101-1[a,c] 162-3[a] 719-3[a,c] 226-11[a] 240-3[a] 46D2[b] 52A4[b] 49C2[b]	7B1[c] 19C5[c] 24A1[c] 25A1[c] 25A2[c] 25B4[c] 45C5[c] PVE[c] PVK[c]	CVS-11 ERA	**(Lafon *et al.*, 1983)**[a] **(Prehaud *et al.*, 1988)**[c] **(Benmansour *et al.*, 1991)**[b]
III	330-338	mAb mapping	194-2[a,d,e] 248-8[a,d,f,e] 718-4[a] 507-1[a,e] 50AC-1[f] 50AD-1[f] 21B4[e]		CVS-11	**(Lafon *et al.*, 1983)**[a] **(Dietzschold *et al.*, 1983)**[d] **(Seif *et al.*, 1985)**[e] **(Coulon *et al.*, 1998)**[f]
IV	251 or 263-264	mAb mapping	110-3[g] RG129[h] 15-15[i]		CVS-11 ERA HEP-Flury RC-HL	**(Bunschoten *et al.*, 1989)**[g] **(Ni *et al.*, 1995)**[h] **(Luo *et al.*, 1997)**[i]
a	342-343	mAb mapping	40E1[b] 48C5[b] 40A1[b] 49C3[b] 20A2[b] 11C6[b]		CVS-11	**(Benmansour *et al.*, 1991)**[b]

Table 1.4: Amino acid sequence of the lyssavirus antigenic sites. The amino acid sequences are given (numbering starts at the beginning of the ORF). The residues coloured in red are those that differ from the RABV sequence, given in the top row. Taken from (Evans *et al.*, 2012).

Virus	Phylo-group	Site II b (53-61)	Site II a (217-219)	Site I (245-250)	Site IV (282-283)	Site III (349-357)	Site 'a' (361-362)	Accession Number
RABV	I	GCTNLSEFS	KRA	KLCGVL	FH	KSVRTWNEI	KG	RABV_CVS GQ918139.1
ABLV	I	GCTSLSGFS	KKA	KLCGIS	FN	KSVRTWDEI	KG	ABLV AF418014.1
ARAV	I	GCTNLSGFT	KKA	KLCGVM	FH	KSVREWTEV	KG	ARAV EF614259.1
BBLV	I	GCTTLTVFS	KKA	KLCGVS	FH	KSIRQWTEI	KG	BBLV JF311903.1
DUVV	I	GCTTLTPFS	KKA	RLCGIS	FH	KSVREWKEI	KG	DUVV_RV131 AY062055.1
EBLV-1	I	GCTTLTPFS	KKA	RLCGVP	FH	KSVREWKEV	KG	EBLV-1_RV20 AY062061.1
EBLV-2	I	GCTTLTVFS	KKA	KLCGIS	FH	KSIREWTDV	KG	EBLV-2_RV628 AY721613.1
IRKV	I	GCTTLTAFN	KKA	KLCGMA	DR	KSIREWKEI	KG	IRKV EF614260.1
KHUV	I	GCTTLSGFT	KRA	KLCGVS	FH	KSIREWSEI	KG	KHUV EF614261.1
LBV A	II	GCSETSSFT	RKA	TLCGKP	NR	KRVDNWVDI	KG	LBV_RV767
LBV B	II	GCGTSSVFS	KKS	TLCGKP	NR	LRVDSWNDI	KG	LBV_NIG1956 EF547431.1
LBV C	II	GCSDTATFS	KKS	TLCGKP	NR	LRVDSWNDI	KG	LBV_RV3 AY062049.1
LBV D	II	GCSTSTSFS	RKA	TLCGKP	NR	RRVDNWTDI	KG	LBV_KE576 GU170202.1
MOKV	II	GCNAETPFT	QKA	TLCGKP	DR	KRVDKWTDI	KG	MOKV_RV4 AY062051.2
SHIV	II	GCSSSSTFS	KKS	TLCGKP	NR	KRVDRWEEI	KG	SHIV GU170201.1
WCBV	III	YCTTEQSIT	KLV	SICGRQ	IK	IKVENWSEV	KG	WCBV EF614258.1
IKOV	III	GCNEGSKVS	ILL	IICGKS	VK	KSVDNWTDI	PI	IKOV JN800529

is this tertiary structure which enables recognition by antibodies or neuronal receptors (Benmansour *et al.*, 1991).

The location of antigenic site IV is currently undefined. Studies have suggested that site IV is present at residues 263-264 so consists of only two amino acids and is continuous though it contains overlapping linear epitopes (Ni *et al.*, 1995). It has also been suggested that site IV contains overlapping linear epitopes with key residues at 251 and 264 (Both, 2013) though another study suggested that site IV comprises only of residue 251 (Liu *et al.*, 2013a). This suggests that a more conclusive investigation into the exact location of antigenic site IV may be required. For the purposes of this investigation, antigenic site IV has been defined as residues 263-264. Minor site 'a' is located in close proximity to site III but contains no overlapping epitopes and consists of only two amino acids (Benmansour *et al.*, 1991).

1.1.2.5 The Large Polymerase

The polymerase (L) is the largest of the rhabdoviral proteins at around 2142 amino acids and it represents 54% of the viral genome (Koser *et al.*, 2004). It is responsible for the viral transcription activities with four main functions: as an RNA dependent RNA polymerase (RdRp), mRNA methyltransferase, mRNA guanylyltransferase and a poly(A) synthetase (Wunner, 1991). The overall sequence of rhabdoviral L proteins is fairly homologous to other *Mononegavirales* including vesicular stomatitis virus (VSV) and Sendai virus however some regions show considerable variability, likely due to the multifunctional nature of L (Poch *et al.*, 1990).

1.1.2.6 Genomic Diversity

A great deal of diversity between lyssavirus genomes has been observed and two elements of lyssavirus virology that determine variation are the presence of defective interfering (DI) particles and viral quasispecies. Virus quasispecies are variant RNA species present in all RNA virus populations and are generated as a result of the error prone nature of the RNA dependant RNA polymerase (Vignuzzi *et al.*, 2006). Virus species that contribute to a virus population may include those with single mutations, those with multiple mutations and those that have had complete deletions of stretches of the genome; DI particles (Finke & Conzelmann, 1999; Wunner & Clark, 1980). The latter are believed to only be generated in isolates following extensive *in vitro* passage but can contribute significantly to pathogenesis (Perrault, 1980). The former will, no doubt affect viral fitness but as yet determining the dynamics of how this occurs remains to be defined.

The role of single/multiple mutations within dominant virus populations have been investigated in several studies. Growth of a human RABV isolate in cell culture was investigated and significant intrinsic heterogeneity within the glycoprotein sequence was observed (Benmansour *et al.*, 1992). When comparing a dog derived street RABV isolate with CVS, a 10% divergence in amino acid composition was observed; however, in the ectodomain there was only a 6% divergence which may suggest that some structural features may restrict divergence in this region. Additional evidence comes from sequencing of viral clones; only one third of clones isolated from the brain of a rabid dog exhibited the consensus sequence, the remaining two thirds showed between one to three amino acid substitutions. It is suggested that the nature of these heterogeneous populations enables the virus to rapidly adapt to changes in the

environment. Deep sequencing techniques are increasingly being used to analyse the full range of sequences present in a viral population to further our understanding of variation among viral quasispecies.

1.1.3 Life Cycle

Rabies is most commonly transmitted via infected saliva transfer from an animal bite. It is thought that the virus initially replicates in muscle cells at the site of the wound (Bellinger *et al.*, 1983) then is able to enter either motor or sensory nerves to travel by retrograde transport along axons to the central nervous system (CNS) (Lo *et al.*, 2001; Mrak & Young, 1993). Virus RNPs are able to travel along axons in a retrograde fashion at a rate of 50-100mm/day (Tsiang *et al.*, 1991). The mechanism of axonal transport may in part be due to interactions between the P protein and actin and microtubule networks including the microtubule motor molecule, dynein LC8 (Jacob *et al.*, 2000; Raux *et al.*, 2000). The virus is able to spread rapidly within the CNS due to the ubiquitous nature of the proposed receptors whilst movement in the periphery appears limited which restricts the spread of virus in these areas (Ugolini, 2008). Once the virus reaches the brain it continues to replicate and spread through the tissue via cell to cell transmission or trans-synaptic spread. From the brain the virus is able to travel along neurones to the salivary glands for onward transmission and also to many other areas of the body (Jackson *et al.*, 1999).

1.1.3.1 Adsorption

The lyssavirus glycoprotein is the main viral protein that enables virion attachment and entry into host cells. Currently, whilst there are postulated to be as yet undefined receptors utilised by lyssaviruses for cell entry, several receptor molecules have been

proposed to be involved in virus binding and entry (Lafon, 2005). Once the virus has entered the host it may either enter the nervous system directly or may initially replicate in the muscle tissue surrounding the locus of infection before entering the nervous system. The exact sites of virus replication prior to entering the central nervous system (CNS) are yet to be defined, however, the suggestion of low level replication in the non-neuronal tissue following infection may provide some explanation for the long incubation periods which have been reported (Lafon, 2005). It has been demonstrated *in vitro* that the virus is able to enter motor nerves via neuromuscular junctions (NMJs). As well as this, viral antigen has been shown to be present in proprioceptors, sensory spindles and stretch receptors *in vitro* (Lewis *et al.*, 2000).

The virus is able to enter neural tissues via one/a combination of three proposed receptors which are present in all mammals: the neural cell adhesion molecule (NCAM) which is present in NMJs at post-synaptic membranes (Thoulouze *et al.*, 1998), the nicotinic acetylcholine receptor (nAchR) which is involved in interneuronal signalling within both the peripheral and central nervous systems (Gastka *et al.*, 1996) and p75NTR which is a neurotrophin receptor responsible for apoptosis, axonal elongation and synaptic transmission (Dechant & Barde, 2002). Each of these three proposed receptors are not only expressed in the CNS but can be expressed on cells in the dermis; NCAM and p75NTR are expressed from dermal fibroblasts associated with hair follicles (Botchkareva *et al.*, 1999; Muller-Rover *et al.*, 1998) whilst nAchR has been detected in the squamous epithelium (Arredondo *et al.*, 2002). This suggests that they may be available around sites of infection as a route to the CNS for the virus; however the extent to which rabies and the lyssaviruses utilise each of these proposed receptors is undefined.

1.1.3.2 Penetration and Uncoating

Upon attachment of the virus to a permissive cell it proceeds to enter via fusion of the viral envelope with the plasma membrane of the cell (Wunner & Conzelmann, 2013). The virus is also able to enter cells through attachment and engulfment by coated pits (viropexis) or via internalisation of vesicles (pinocytosis), both of which can result in the internalisation of multiple virions simultaneously (Tsiang, 1993). Once internalised the G protein initiates fusion of the viral envelope with the endosomal membrane to release the RNP into the endosomal vesicles (Wunner & Conzelmann, 2013). Lysosomes then fuse with these vesicles to release the RNP into the cell cytoplasm (Gosztonyi, 1994) to initiate transcription (Finke & Conzelmann, 2005b).

1.1.3.3 Primary Transcription

The lyssavirus genome consists of a strand of linear, negative sense, single stranded RNA. This means virus replication requires transcription of mRNA. The L protein, as an RdRp initiates transcription of the genomic RNA to produce leader RNA which is an exact complement of the 3' end of the genome RNA. It is phosphorylated at the 5' end but has no 3' poly(A) tail. Five complementary positive sense mRNAs are also transcribed which are capped at the 5' end and polyadenylated at the 3' end, each corresponding to one of the five viral genes (Banerjee, 1987). The transcripts are generated in consecutive mono-cistronic fashion with the sequential nature of transcription controlled by the transcriptional complex. This complex is able to recognise the start, stop and polyadenylation signals which flank each cistron. A gradient effect of transcriptional efficiency has been observed from the 3' to 5' genes. This process allows structural proteins at the 3' end of the genome e.g. N to be produced in

higher concentrations than the 5' proximal genes (Tordo & Kouknetzoff, 1993).

1.1.3.4 Translation and Protein Processing

The viral mRNAs are translated using the host cell machinery. The N, M, P and L proteins are translated on free ribosomes within the cytoplasm whereas the mRNA encoding G is translated on membrane bound ribosomes (Tordo, 1996). Completion of the G processing is carried out co-translationally: firstly core glycosylation occurs in the rough endoplasmic reticulum (RER) and this is followed by final processing of the carbohydrate side chains in the RER and Golgi body (Shakin-Eshleman *et al.*, 1992). The now glycosylated transmembrane G protein is transported to the cell membrane, the glycosylated ectodomain protruding from the exterior of the cell (Tordo, 1996).

1.1.3.5 Replication

Although the exact mechanisms that drive a switch from transcription to replication remain unclear, at some stage following infection a switch occurs to drive the generation of a positive strand full length genome replicative intermediate. This replication is dependent on continued synthesis of viral N protein. Once a full length antigenome has been produced, this acts as the template for genome replication to produce nascent genome sense RNA. During the switch from primary transcription to genome replication the activity of the viral polymerase changes from a transcriptive to a replicative mode. As the positive sense RNA is synthesised it is immediately associated with the newly generated N protein to form positive sense RNP and it is this which acts as the template for replication. Negative sense genome is then amplified; again with the negative sense RNA being immediately encapsidated by the N protein. Once the negative sense RNP has been amplified this acts as a template for secondary transcription which results in

amplification of all viral proteins for subsequent virion assembly (Banerjee, 1987).

1.1.3.6 Assembly

The process of RABV morphogenesis starts with the formation of the RNP during replication. M then associates with the RNP complex in the cell cytoplasm and down-regulates RNA transcription while up-regulating replication (Finke & Conzelmann, 2003). M initiates tight coiling of the RNP complex and localises the coiled RNP-M at the cell membrane. At the membrane of the cell the M protein bridges the RNP and the cytoplasmic tails of G and as the components assemble the polymerase activity of L is halted by M within the very tightly coiled RNP and virus budding is initiated (Wunner & Conzelmann, 2013).

1.1.3.7 Budding

Once the RNP-M complex has associated with the transmembrane G proteins at the cell membrane, the mature virions bud through the cell membrane, acquiring their lipid bilayer envelope during the process (Wunner & Conzelmann, 2013). The interaction of the G cytoplasmic tail with the M protein within the virion is thought to hold the virus in its classic bullet-like morphology. It is possible that the RNP-M complex is able to bud though the cell membrane in areas where no viral G trimers are expressed and in this case the viral particles produced are non-infectious (Mebatsion *et al.*, 1996a). It has also been observed that virions may bud through the endoplasmic reticulum or Golgi body within the cells (Lahaye *et al.*, 2009) into the lumen of vesicles produced by these membranes. In this case it is possible that these vesicles would be secreted from the infected cells by the normal secretory pathway (Wunner & Conzelmann, 2013).

1.1.4 Epidemiology

RABV is distributed globally with only a few, principally island nations, being free of disease. Concerted vaccination campaigns with domestic animals across both the USA and Western Europe have now eliminated the burden of rabies from domestic terrestrial carnivore populations. In the USA, sylvatic rabies remains a constant threat to human populations with racoons, skunks and foxes acting as reservoirs for the classic rabies viruses but with bats causing increasingly more human infections (Blanton *et al.*, 2011). Extensive wildlife vaccination campaigns have managed to eliminate sylvatic rabies from Western Europe. However, globally the existence of lyssaviruses circulating in Chiropteran reservoirs remains and so a complete eradication of rabies virus is thought to be unrealistic (Rupprecht *et al.*, 2008). Within the USA, bat species maintain RABV whilst, in contrast across the Old World, classical RABV appears to be totally absent from bat populations whilst several non-rabies lyssaviruses persist. These populations of bats are very difficult to access, and across the EU, are protected by law (Freuling *et al.*, 2012) making eradication of lyssaviruses an unfeasible target.

From an epidemiological perspective, within Europe, alongside RABV in terrestrial wildlife populations three other lyssavirus species have been genetically characterised: European Bat Lyssavirus type-1 (EBLV-1), European Bat Lyssavirus type-2 (EBLV-2) and Bokeloh Bat Lyssavirus (BBLV).

EBLV-1 is genetically classified into two lineages; EBLV-1a is found throughout northern Europe and EBLV-1b has been detected in southern Germany, France and Spain. Both EBLV-1 lineages are associated with infection of *Eptesicus fuscus*, the Serotine bat. Infection of other bat species such as the Meridional Serotine (*E.*

isabellinus) may suggest the circulation of a third lineage of EBLV-1 (Banyard *et al.*, 2011; Vazquez Moron *et al.*, 2011). EBLV-1 cross species transmission (CST) events have occurred at a very low frequency although to date independent terrestrial transmission pathways have not been established (Dacheux *et al.*, 2009; Muller *et al.*, 2004; Tjornehoj *et al.*, 2006).

In contrast, EBLV-2 is predominantly associated with infection of *Myotis daubentonii*, the Daubenton's bat, across much of northern Europe and no cross species transmission events have been reported in terrestrial species although two fatal human cases have occurred (Fooks *et al.*, 2003; Lumio *et al.*, 1986).

The first isolate of BBLV was obtained from a Natterer's bat (*Myotis natterei*) in Germany in November 2009 (Freuling *et al.*, 2011). Since then two further detections have been reported including another in Germany (Freuling *et al.*, 2013) and an isolate from France (Picard-Meyer *et al.*, 2013). Evidence for a further lyssavirus has been reported in Spain in a common bent-wing bat (*Miniopterus schreibersi*). Although only a short stretch of genetic data has been determined for this virus it has been named Lleida bat virus (LLEBV). Initial sequencing of the N gene suggests considerable genetic divergence from the current European lyssaviruses (Arechiga Ceballos *et al.*, 2013).

A further four Eurasian lyssaviruses have been described, predominantly from single virus isolates from different bat species. These include Aravan virus (ARAV) isolated from a Lesser-mouse Eared bat (*Myotis blythi*) in Kyrgyzstan (Botvinkin *et al.*, 2003), Khujand virus (KHUV) isolated from a whiskered bat (*Myotis mystacinus)* in Tajikistan (Kuzmin *et al.*, 2008b), Irkut virus (IRKV) isolated from a Greater Tube-nosed bat

(*Murina leucogaster*) in Eastern Siberia (Botvinkin *et al.*, 2003) and West Caucasian Bat virus (WCBV) isolated from a common bent-wing bat (*Miniopterus schreibersi*) in the Caucasus mountains in Russia (Botvinkin *et al.*, 2003). Phylogenetic classification of these Eurasian isolates has shown them to be distinct species within the lyssavirus genus with WCBV being the most genetically divergent (Horton *et al.*, 2010). Of these, only Irkut virus has been isolated on more than one occasion (Liu *et al.*, 2013b) and has been associated with human fatality (Belikov *et al.*, 2009).

In other parts of the world several other, predominantly bat associated lyssaviruses occur (Banyard *et al.*, 2011). In Australia, Australian bat lyssavirus (ABLV) has been isolated from five different bat species since its initial isolation in 1996 (McCall *et al.*, 2000) and has also been implicated in three human fatalities (Allworth *et al.*, 1996; Hanna *et al.*, 2000; Weir *et al.*, 2013). There have also been two confirmed cases of horse infection with ABLV in Queensland (Symons, 2013) which have raised concerns about a theoretical yet possible risk of transmission of ABLV infection from horses to humans.

Across Africa a number of bat lyssaviruses have been detected. The most frequently reported is Lagos bat virus (LBV) which has been isolated from multiple bat species distributed across sub-Saharan Africa (Hayman *et al.*, 2008). There are currently four defined lineages of LBV; lineage A consists of Kenyan (2007), Senegalese (1985) and a French (1999) isolates, lineage B contains only the original Nigerian (1956) isolate, lineage C contains isolates from Zimbabwe, South Africa and the Central African Republic and lineage D contains a single isolate identified in an Egyptian fruit bat in Kenya (Kuzmin *et al.*, 2010; Markotter *et al.*, 2008). It has been suggested that Lagos bat virus isolates could be subdivided into several genotypes or lineages based on

criteria adopted by the ICTV whereby all isolates belonging to the same species have identities of greater than 80-82%. LBV isolates can be more divergent than this, within the variable glycoprotein, across each lineage and as such may ultimately be re-classified.

Duvenhage virus (DUVV) was initially isolated from a fatal human infection following a bat bite and was subsequently isolated from a number of bat species, mainly Nycteris species in South Africa and Zimbabwe. There have been two further cases of DUVV infection in humans, both of African origin (Paweska *et al.*, 2006). Only a single isolate of Shimoni bat virus (SHIBV) exists and was isolated in 2009 from a *Hipposideros vittatus* bat in Kenya (Kuzmin *et al.*, 2010).

Mokola virus (MOKV) is an African lyssavirus which is only one of two lyssavirus species that have not been detected within bat species. MOKV has been isolated from a range of mammals including a shrew, domestic cats and a dog. Most of the isolates have been from South Africa and neighbouring Zimbabwe but there have also been sporadic isolations from Nigeria, Cameroon, Central African Republic and Ethiopia. Interestingly, sera from LBV positive bats have been shown to neutralise MOKV making an assessment of the range of species infected with MOKV difficult and as a result bats cannot be ruled out as a reservoir for MOKV (Kgaladi *et al.*, 2013). There have been two recorded human cases of MOKV infection (Familusi & Moore, 1972; Familusi *et al.*, 1972).

The most recent African lyssavirus to be identified is Ikoma lyssavirus (IKOV). IKOV was isolated from an African civet and is the second non-rabies lyssavirus to have been

isolated only from a terrestrial carnivore species although its origin within Chiropteran species is assumed from the circumstances within which it was detected (Marston *et al.*, 2012b). IKOV is genetically divergent from all the other lyssaviruses (Marston *et al.*, 2012a).

1.1.5 Infection with lyssaviruses

Several aspects of lyssavirus virology remain unclear with the events that lead to either an established infection and death, or so called abortive infections being largely undefined. Alongside this, recent studies have suggested that in some instances infection and clearance by an undefined immune mechanism may also occur (Gilbert *et al.*, 2012). What is understood following an exposure to a lyssavirus is detailed below.

1.1.5.1 Transmission

The most common route of infection with a lyssavirus is via the mechanistic action of a bite from an infected animal which contains live virus in its saliva. Historically, this mechanism was first described over 200 years ago by experimental work in 1804 by Zinke (Zinke, 1804). Alongside this early observation, it was later determined that these viruses are unable to cross an intact dermal barrier. However, if the skin is broken, the risk of infection is greatly increased. Lyssaviruses can also infect hosts via mucous membranes including the nasal lining, oral cavity, conjunctivae, external genital organs and the anus. There have also been reports of infection via inhalation of virus via the aerosol route (Constantine, 1962; Johnson *et al.*, 2006) although conclusive evidence to validate this route of infection is lacking. Human-to-human transmission has only been reported very rarely with transmission of bodily fluids being implicated as the source of virus (Fekadu *et al.*, 1996). Alongside this, there have been occurrences of infection

through transplantation of infected tissue. A case in 2004 resulted in three rabies fatalities when an infected donor supplied a liver section, a kidney and a section of artery to three separate recipients. Within 30 days of transplantation all three recipients had returned to the hospital with rapidly progressing neurological disease, which was diagnosed post mortem as rabies infection (Burton *et al.*, 2005). A further case in Germany resulted in the deaths of three individuals following organ donation (Johnson *et al.*, 2005). Corneal transplantation has also facilitated infection in the recipient following surgery (Javadi *et al.*, 1996).

1.1.5.2 Incubation Period

The incubation period of rabies virus is widely accepted to be between 20-90 days, however, there have been reports of extreme cases of incubation periods ranging between 14-19 years although in these cases the possibility of a secondary infection cannot be discounted (Fishbein, 1991). Recent human cases in the UK have highlighted incubation periods of up to two and a half years (Hunter *et al.*, 2010). Regardless, it is clear that the incubation period following infection can vary greatly depending on a range of factors. Due to the neurotropic nature of the virus, a bite/site of infection that is highly innervated (e.g. face and hands) can lead to a much shorter incubation period or faster progression of disease than infection at a less densely innervated area. Alongside this, the presence/absence of permissive receptors in the tissue surrounding the infection site can affect viral entry and spread, hence increasing the incubation period. Muscular nicotinic acetylcholine receptors (a proposed receptor for rabies virus) have been identified at post synaptic muscle membranes which may enable the amplification of virus before it enters the nervous system (Lafon, 2005). The dose of infective virus also plays a role in determining incubation period (Ugolini, 2008). As well as this the

immune status and age of an individual can influence incubation as incubation periods in paediatric cases are shorter than those in adults, presumably due to their smaller stature (Warrell, 2008).

Incubation periods following exposure to non-rabies lyssavirus are also poorly defined as possible exposure times are often difficult to determine. In particular it is recognised that bat bites may go totally unnoticed leading to cryptic cases of rabies (Messenger *et al.*, 2003). A case of EBLV-1 infection in a human recorded an incubation period of 45 days (Botvinkin *et al.*, 2006). Further to this, a case of EBLV-2 infection in a bat handler reported a 19 week incubation period, however, the patient had a documented history of bat bites which may have confounded determination of the incubation period (Fooks *et al.*, 2003). DUVV infection has been reported to have an incubation period of around 4 weeks (Meredith *et al.*, 1971; Paweska *et al.*, 2006) whereas ABLV may have a much longer incubation period as a case in 1998 reported onset of clinical disease 20 months after exposure to a bat (Hanna *et al.*, 2000; Johnson *et al.*, 2008). Interestingly, infection of bats with lyssaviruses has also suggested inordinately long incubation periods. For example, a Daubenton's bat kept in isolation in a bat rehabilitation centre was seen to be asymptomatic for a period of 9 months before developing aggressive behaviour that was diagnosed, following termination, as EBLV-2 infection (Pajamo *et al.*, 2008).

The principal mechanisms of lyssavirus pathogenesis within the peripheral and central nervous system (P/CNS) remain poorly defined and mice brains infected with rabies virus show little morphological damage (Song *et al.*, 2013). Once in the CNS the virus may alter neurotransmission and normal neural function. It has been demonstrated that there is a reduction in the expression of housekeeping genes in RABV infected neurones

(Prosniak *et al.*, 2001). This results in a reduction of protein synthesis and subsequently affects neuronal activity. It has also been suggested that the presence of the G protein on the surface of infected cells may disrupt ion channels, supported by the observation of electrophysiological disturbances in RABV infected murine brains (Dietzschold *et al.*, 2005).

1.1.5.3 Immune Response

The first line of defence against infection is the innate immune response. This response has been shown to have little impact in rabies infection (Chopy *et al.*, 2011). Initially, during migration from the periphery to the CNS, rabies activates the innate immune sensors retinoic acid-inducible gene I (RIG-I) and melanoma-associated gene 5 (MDA-5), both of which are cytoplasmic helicases (Loo *et al.*, 2008). RIG-I recognises and binds to both ssRNA and dsRNA whereas MDA-5 recognises long dsRNA fragments (Jensen & Thomsen, 2012). These interactions induce the recruitment of intracellular adaptor proteins which initiate transcription and expression of type I interferon (IFN) (Kawai & Akira, 2008). The type I IFN response is generally responsible for inducing an anti-viral environment within the infected host however rabies has developed a number of mechanisms to circumvent the type I IFN response, thus abrogating the impact of the innate immune response in rabies infection (Chopy *et al.*, 2011). The IFN response upregulates the expression of FasL and B7-H1 on migratory T cells. These molecules are responsible for limiting the effects of migratory T cells within the CNS as they induce apoptosis in T cells expressing the corresponding ligands: PD-1, Fas and CD8. The IFNs released in response to RABV infection upregulate the expression of FasL and B7-H1 on migratory T cells which enhance apoptosis of T cells both within the periphery and the CNS of the infected host, thus enhancing survival of rabies virus (Lafon, 2011).

In addition to the RIG-I like receptors (RLRs) mentioned, toll-like receptors (TLRs) also respond to the presence of pathogen associated molecular patterns (PAMPs). TLR3 is of particular significance in rabies infection as it recognises both dsRNA and the envelope glycoprotein (Jensen & Thomsen, 2012). TLR3 is found in intracellular compartments of both dendritic cells (DCs) and neuronal cells so is able to recognise viruses which enter cells via the endocytic pathway, as RABV does. During the infection process, TLR3 recognising its ligand would result in the activation of various transcription factors including NF-κB which would result in downstream activation of IFN-α/β, however, during RABV infection TLR3 is sequestered into perinuclear inclusion bodies within infected neurones (Menager *et al.*, 2009). This sequestration of TLR3 results in a reduction of innate immune response activation as well as a reduction in the activation of apoptosis of infected cells (Lafon, 2011), all of which combine to promote the survival of RABV in the infected CNS.

Due to the invariably 100% fatality rate of rabies infection once clinical symptoms present, a pre-exposure immunisation is recommended for all those at risk of infection including scientists working with the virus, bat workers and travellers to endemic areas. Four days after vaccination immunoglobulin M (IgM) becomes detectable and this is followed by the IgG response three days later. It has been shown that Ig responses persist up to two years and it is IgG which provides the most effective level of protection. This is likely due to IgM being unable to penetrate tissue (Johnson *et al.*, 2010; Turner, 1978).

The vital importance of neutralising antibodies in a protective immune response was demonstrated using gene knockout mice. Those lacking B cells and those lacking both T

and B cells were susceptible to attenuated strains of RABV whereas those lacking only CD8 T cells were not susceptible to infection (Hooper *et al.*, 1998). In humans, detectable antibody levels do not generally develop until days after the onset of clinical disease. It was found that out of eleven human cases studied in Thailand, only three produced detectable, yet low, neutralising antibody levels. The cerebrospinal fluid (CSF) of six of these patients was investigated yet none contained detectable antibody (Kasempimolporn *et al.*, 1991). Similarly, in a sample of twenty rabies cases in the USA between 1980 and 1996, none had detectable antibody levels upon presentation to hospital however more than 50% of these patients had generated detectable titre within ten days from admission. In addition, just two of fourteen selected patients from this sample showed detectable antibody levels in the CSF (Noah *et al.*, 1998). A similar pattern has been observed in two documented RABV cases imported into the UK. One patient did not develop detectable serum antibody until sixteen days after hospital admission (Solomon *et al.*, 2005) whereas the second patient developed antibodies in both the serum and CSF however this did not occur until the second week post hospital admission (Johnson *et al.*, 2010). An indigenous case of EBLV-2 infection in the UK did not develop any detectable antibodies throughout the course of clinical infection (Nathwani *et al.*, 2003).

It is likely that this late development of neutralising antibodies in the periphery and therefore a lack of antibody response at the site of infection combined with low to non-existent levels of neutralising antibody in the brain, where it is most needed, contribute to the devastatingly high degree of mortality in rabies infections (Johnson *et al.*, 2010).

1.1.5.4 Human Infection

In human infection, where disease progression has been characterised, the development of clinical disease following lyssavirus infection is indistinguishable from that caused by RABV. Indeed, in areas where RABV is endemic, a patient presenting with idiopathic neurological symptoms should not have rabies ruled out and an absence of bite history should not rule out the possibility of rabies in a differential diagnosis (Rupprecht, 1996). It has been shown that misdiagnosis of neurological disease occurs with misdiagnosis of rabies as malaria being one example (Mallewa *et al.*, 2007). Although infection via a bite from a rabid animal is the most common route of infection other routes must be considered; infection may occur through infected saliva contact with exposed scratches or an open wound as well as exposure with mucous membranes.

The 2-20 day prodromal phase of a RABV or lyssavirus infection are easy to confuse with many other diseases as the patient often presents with weakness, malaise, fever, headaches, urinary problems and paraesthesia at the site of infection which leads to ascending paralysis. For this reason other diseases such as Guillain-Barre syndrome are included in a differential diagnosis (Grattan-Smith *et al.*, 1992; Plotkin, 2000). From the point of clinical onset, the likelihood of recovery is effectively zero. Approximately 80% of individuals then go on to develop the classic ‘furious’ form of rabies that can be characterised by hydrophobia and respiratory convulsions whilst the remainder may develop a ‘dumb’ or ‘paralytic’ form of disease (Toacsen & Moraru, 1985). The reason for these different clinical courses is not yet defined but may be related to the source of infection i.e. a low level dermal bite from a bat versus a deep intramuscular bite from a dog, genetics of the host or genetics of the virus itself. Death most often results from respiratory failure as a result of the spasms brought on by hydrophobia or cardiovascular

failure (Leung *et al.*, 2007).

1.1.5.5 Animal Infection

It appears that there are no species specific symptoms but similar to human infection, there is a prodromal phase where the animal appears lethargic and exhibits some weakness. It has been observed that domesticated animals may show less interest in their owners whereas wild animals may actively seek contact with humans (Niezgoda *et al.*, 2002). Animal infection also follows the dumb or furious forms of disease but individuals may present with features of both. Clinical progression may be linked to the site of viral replication within the CNS which may be linked to the site of infection and the dose of virus transmitted (Healy *et al.*, 2013b). In animals exhibiting signs consistent with furious rabies there is often a short prodromal phase where the animal may appear to have heightened alertness and this is followed by a neurological phase with signs similar to those seen in human infection. Rabid animals have been observed to become hyper-reactive to external stimuli, including noise, light or touch and may attack other humans, animals or inanimate objects. It is presumed that animals also experience paraesthesia at the site of infection as excessive grooming and scratching has been observed which can lead to self-mutilation. Death results from organ failure and respiratory arrest. Paralytic rabies in both domesticated and wild dogs starts with a clinical presentation of lethargy and a lack of coordination. This leads to weakness in the hind limbs and subsequent motor paralysis that spreads eventually to the mandible which results in cessation of the swallowing reflex. This is the final stage of disease before death (Hanlon, 2013).

1.1.5.6 Seropositivity to rabies in mammals

With infection, following the onset of clinical disease, leading invariably to death the presence of healthy seropositive animals remains a quandary. The question of whether bats possess a level of tolerance to rabies infection/disease has often been raised and numerous studies have detailed high levels of seropositivity in bats; for example a study of Brazilian free-tailed bats showed that 69% of those sampled had virus neutralising antibodies (VNA) to rabies virus but only 0.5% had active infection. This was determined by direct fluorescent antibody testing of brain samples (Steece & Altenbach, 1989). This suggests that seroconversion may be common in natural infections but it is still unclear whether these infections lead to any CNS involvement which is subsequently resolved.

In addition to seroconversion in bats, there have been multiple documented cases of seroconversion in other animals. A study in hyenas in the Serengeti over a period of 9 to 13 years showed that 37% were seropositive with a further study following some of these hyenas demonstrating that half of these seropositive animals lost their VNA titres over time. The higher ranked animals tended to have high VNA and this may be due to the observed high bite rates and oral contacts with other hyenas involving lower ranked animals licking the open mouths of the higher ranked (East *et al.*, 2001). During the period of the study, no clinical rabies disease was observed and no virus could be isolated from the saliva of the seropositive animals. The saliva did however yield RT-PCR positive results for rabies RNA in half of the seropositive cases. Sequence analysis of the RNA isolated from the hyena saliva showed that the sequences were most closely related to dog strains in the Middle East and Europe. They were divergent compared to the commonly circulating strains of rabies virus in other mammals in the Serengeti.

Overall this suggests that there may be a role for low virulence strains of rabies in pathogenesis which may not cause clinical disease.

In support of this evidence from animals there has been a recent documented case of seropositivity in humans. A study of two isolated communities in Peru looked at possible risk factors for rabies infection via a questionnaire and isolation of blood samples from participants. It was found that 11% of participants had VNA against rabies virus and 86% of these seropositive individuals reported having been bitten by a bat with only one seropositive individual having received PEP. This suggests that there may have been a number of non-fatal infections in these communities, most likely by vampire bats which circulate in these areas and are reported to feed off cattle kept by these communities (Gilbert *et al.,* 2012).

The findings of these studies are in opposition of the currently commonly held view that rabies causes clinical disease and death in cases of successful infection and suggest that further investigation into seropositivity in mammals must be conducted before these phenomena can be fully understood.

1.2 Current Prophylactic and Therapeutic tools

1.2.1 Human Interventions

The lyssaviruses represent the only group of viruses for which, following the development of clinical disease, the outcome of infection is invariably fatal (Fooks *et al.*, 2014). This feature of these viruses has made rabies virus one of the most feared infections in human history. Interestingly, the development of a medical intervention that ultimately involves the induction of a therapeutic coma has resulted in a small

number of cases where individuals have survived infection, albeit with permanent neurological sequealae (Jackson, 2013). This treatment, termed the Milwaukee Protocol (MP) where success has been seen, appears to work by reducing the activity of the patient's nervous system which is hypothesised to enable the immune system to catch up with the progress of the virus through the nervous system and to neutralise the spread of virus (Willoughby *et al.*, 2005). Whilst initially heralded as a breakthrough in the battle against rabies virus, the procedure has so far had a low survival rate with between one and three individuals surviving beyond 4 years without neurological sequelae (Jackson, 2013; Willoughby, 2009).

1.2.2 Pre and post exposure tools

The continued failure of the MP to save individuals that have developed clinical rabies means that currently the only tools to prevent death prior to the development of clinical disease are vaccination and rabies immunoglobulin. Historically, nerve tissue based vaccines (NTVs) were used that were generated from desiccated brain and spinal cord material from infected animals. The early vaccines could stimulate neuroallergenic responses in vaccinees which was problematic although later these live vaccines were inactivated with β-propiolactone to reduce reactogenicity (Bugyaki *et al.*, 1979). Some reports suggest that these vaccines are still in use in some rabies endemic countries (Parviz *et al.*, 2004) though the WHO advises against the use of these reactogenic vaccines. The advent of tissue culture techniques for the generation of vaccines led to the development of the human diploid cell vaccine (HDCV) (Wiktor *et al.*, 1964) which involved adapting classic RABV strains to growth in human cell culture then subsequent inactivation. The HDCV was able to produce a highly protective immune response in most vaccinees but had high production costs which inhibited its use in many of the

developing countries that needed it most. Purified chick embryo cell vaccines (PCECV) and purified Vero rabies vaccines (PVRV) have been included in a number of studies using Intradermal (ID) vaccination regimens to reduce the cost of PEP (Ambrozaitis *et al.*, 2006; Briggs *et al.*, 2000).

Vaccines can be used as pre-exposure immunisation for individuals at high risk of infection including veterinarians, laboratory workers and those travelling to rabies endemic countries as well as following potential exposure. The OIE and WHO specify that scientists working with rabies viruses must have a minimum antibody titre of 0.5IU/ml post immunisation in order to be protected from accidental infection with classical rabies strains with boosters being recommended should an individual's titre drop below this threshold. Additionally, for other at risk groups including bat workers, regular serological testing and boosters when necessary are recommended (OIE, 2008).

One potential drawback with the current RABV vaccines, however, is that they are unable to induce neutralising antibody responses that can protect against infection with all of the lyssaviruses. Whilst perhaps not significant for the global fight against rabies virus, the requirement for a vaccine that protects against all lyssaviruses is of importance for those at high risk from infection. Alongside those at high risk, the epidemiology of the non-rabies lyssaviruses is poorly understood and so the threat to human life has not been determined. The current RABV vaccines for both human and animal use are based on inactivated preparations of phylogroup I rabies strains. Studies have shown that with these vaccines little to no cross protection is afforded against phylogroup II or III viruses meaning that for a number of lyssaviruses there is no vaccine protection (Badrane *et al.*, 2001; Fooks, 2004; Hanlon *et al.*, 2001). A number of studies have looked into viable

cross protective vaccine strategies, for example it has been shown using recombinant vaccinia viruses expressing the MOKV and WCBV G proteins that protection can be obtained for both viruses, both singly and in combination (Weyer *et al.*, 2008). This shows that there may be potential to generate a pan-lyssavirus vaccine in the future (Evans *et al.*, 2012; Fooks, 2004). In addition to pan-lyssavirus vaccines there is also ongoing development of live attenuated vaccines for use in human post exposure prophylaxis in order to avoid the need for multiple vaccinations before a protective titre is reached. An attenuated triple G variant of rabies has been generated which could potentially be used for human vaccination. The risk of the live vaccine reverting to virulence is negligible due to the inherent safety of the triple apathogenic G which has been shown to be dominant over the pathogenic G in co-infection studies (Faber *et al.*, 2009; Faber *et al.*, 2002). In the meantime, while these vaccines are under development, intra dermal regimens are available which utilise the existing rabies vaccines but use 60% less vaccine and are safer and more practicable than intramuscular methods (Warrell *et al.*, 2008).

Another feature of the existing rabies vaccines is that they can be used to protect against disease after exposure to the virus but before the development of clinical disease. The regimen used once an individual has experienced a potential exposure to virus is termed post exposure prophylaxis (PEP) and includes vaccination coupled with administration of rabies immunoglobulins (RIG). PEP should be administered as soon after the exposure as possible, to reduce the likelihood of the virus establishing a productive infection. Initially, in the case of a bite or scratch the WHO recommends washing the wound thoroughly with soap and water, and it is thought that this simple action can increase survival by 50% (Rupprecht & Gibbons, 2004). Once the wound has been

thoroughly washed it is advisable to soak the site in 70% ethanol or iodine. It is important that the wound is not sutured. The next step is to administer PEP. Rabies immunoglobulins are applied to the wound and any nearby mucous membranes. The immunoglobulins are based on either vaccinated human rabies immunoglobulins (sera (HRIG) or neutralising antibodies generated in horses, equine rabies immunoglobulins (ERIG). HRIG is applied at a dose of 20 international units per kilogram (IU/kg) of body weight and ERIG is applied at a dose of 40 IU/kg. Once the RIG has been applied, a shot of rabies vaccine is administered at a site distant from the wound so that the RIG does not neutralise the virus in the vaccine. There are two main regimens for administration of RIG; the Essen regimen which is practised in Europe and North America and consists of 5 intramuscular doses of vaccine and the Zagreb 2-1-1 regimen which is more commonly used in some parts of Europe as the number of doses of vaccine is reduced compared to the Essen regimen (WHO, 2005). Despite PEP being effective in reducing the incidence of disease it is estimated that it is only used in 5% of cases due to its lack of availability which is related to its almost prohibitive cost (Both *et al.*, 2012). There are however efforts to develop more affordable antibody cocktails to replace RIG, including mouse derived monoclonal antibody (mAb) cocktails (Muller *et al.*, 2009) and plant derived antibodies (plantibodies) (Both *et al.*, 2013).

The WHO has identified five murine monoclonal antibodies (mAbs) which when used in combination have the potential to replace conventional RIG as they are capable of neutralising the same spectrum of lyssaviruses to a comparable titre (Muller *et al.*, 2009). mAbs M777, M727, 1112-1 and E559 were shown to target antigenic site II and 62-7-13 to target antigenic site I on the glycoprotein by sequencing and cross neutralisation experiments. Subsequent investigation into these mAbs as a potential

alternative therapy excluded 1112-1 due to licensing issues but cocktails of two to three of the remaining mAbs are currently under investigation (Muller *et al.*, 2009).

Alongside these therapeutic mAbs there are a number of others which have been identified as strongly neutralising, targeting the lyssavirus glycoprotein. mAb D8 was generated from mouse hybridomas against DUVV and found to strongly neutralise only DUVV *in vitro*, however its neutralisation profile was considered too narrow for inclusion in a therapeutic cocktail (Dr A C Banyard - unpublished observation). mAb D1 was generated in the same manner as D8, via mouse hybridoma against Pasteur virus (PV) strain, a phylogroup I RABV and was found to target antigenic site III of non-denatured rabies glycoprotein (Jallet *et al.*, 1999). mAb D1, like D8 however has also not been considered for use in potential therapeutic cocktails.

1.2.3 Vaccines for domestic and wild animals

The vaccines used in animals, mainly for dogs as these are the species most associated with human infection, have been derived in a similar way to human counterparts. The first vaccines were derived from neural tissue but the reactogenicity of these vaccines drove development of embryonic chicken egg derived vaccines. These egg derived vaccines were effective in adult dogs but could cause clinical rabies in young dogs, cats and even cattle (Bunn, 1991). In order to reduce these vaccine associated deaths, the egg derived passage strains were further passaged in embryonated chicken eggs until, after 205 passages the strain was safe for use intra-muscularly in cats, puppies (>3 months) and cattle (Koprowski *et al.*, 1954). Unfortunately this High Egg Passage (HEP) strain produced a number of vaccine-induced rabies cases in cats and was withdrawn. Current vaccines now recommended for routine parenteral vaccination of domestic animals

include cell culture vaccines (WHO, 2005). These killed vaccines are commonly inactivated with β-propiolactone and the preparations are often combined with other vaccines including canine distemper for dogs and feline parvovirus and calcivirus for cats. Due to the inactivation of the viruses, adjuvants are added to the vaccine preparation to increase the immune response (Dreesen, 2007).

As well as vaccines based on fixed virus strains there are a number of genetically engineered vaccines designed to control rabies in wildlife. A successful oral vaccine composed of vaccinia virus expressing the RABV glycoprotein helped reduce concerns over the safety of live attenuated vaccines (Pastoret *et al.*, 1988). In some parts of Canada, the USA, and across much of western Europe the application of oral vaccination campaigns has successfully eliminated terrestrial rabies which has, in turn, reduced the number of human cases in these areas (Blanton *et al.*, 2011; Krebs *et al.*, 2005). However, in many countries, especially across many parts of the USA, Africa, Asia, Latin America and Eurasia, RABV continues to circulate in certain wildlife populations and development of suitable bait formulations to try and eliminate these pockets of infection continues.

1.3 Utilisation of lentiviral pseudotypes for glycoprotein expression

Lentiviral pseudotype (PT) viruses were initially developed as vectors for gene therapy due to their ability to transduce non-proliferating cells (Naldini *et al.*, 1996). Many of these initial vectors expressed the VSV glycoprotein on the PT surface as this enhanced the stability of the PT particles (Burns *et al.*, 1993). The stability and high titres of these lentiviral VSV PT particles led to the development of the related rhabdoviral lyssavirus PTs, albeit for a very different purpose. Due to the lyssavirus G protein being the sole

target of virus neutralising antibodies (Dietzschold *et al.*, 1982) the expression of this protein alone on the PTs would enable investigation of the role that antibodies specifically targeting the glycoprotein play in a neutralising response (Wright *et al.*, 2008).

Currently, lyssavirus PTs have been utilised to study the neutralisation profiles of a range of vaccinated human and animal sera against a small number of PTs representing CVS, EBLV-1 and EBLV-2. This study showed the utility of PTs for assessing VNAs in sera as an alternative to live virus neutralisation assays (Wright *et al.*, 2008). The specificity and sensitivity of pseudotype neutralisation assays (PNA) in comparison to live virus neutralisation assays has been shown to be comparable and the inclusion of a range of different reporter genes including: luciferase, green fluorescent protein (GFP) and β-galactosidase (*LacZ*) has demonstrated the flexibility of this assay for use in a range of laboratory conditions (Wright *et al.*, 2009). A multiplex PNA of LBV, MOKV and WCBV PTs has also been developed and used to screen a panel of African bat sera to determine seroprevalence against each of these viruses (Wright *et al.*, 2010).

These studies have shown the flexibility and accuracy of PTs to detect VNAs. In addition they have the added advantage of being able to be carried out in lower containment facilities than traditional virus neutralisation tests as PTs are replication deficient as they contain no rhabdovirus genome RNA (Temperton & Wright, 2009).

Pseudotype viruses carry the genome and core of one virus and the envelope of another virus. The benefit of a lentiviral pseudotype is the inherent capability of lentiviruses to reverse transcribe their genomic RNA to create double stranded DNA (dsDNA) which is

then stably integrated into the host cell genome. This means that reporter genes carried by the PT are integrated into infected cells enabling quantification of PT particles by reporter gene expression.

The pseudotype particles are generated by a triple transfection of three plasmids containing the replication complex, glycoprotein and a reporter gene. The retroviral structural proteins including p7, nucleocapsid and the matrix (GAG) and the enzymatic proteins including the protease, integrase and reverse transcriptase (POL) are encoded on the gag-pol plasmid, with the removal of the packaging signal to ensure these genes are not incorporated into the mature virions. The reporter gene plasmid encodes the reporter gene flanked by LTR regions that enable integration and transcription of the PT RNA genome. Upstream of the reporter gene is a packaging signal which ensures this gene is incorporated into the mature virion. The envelope plasmid contains the glycoprotein gene downstream of a promoter sequence. These plasmids are transfected into permissive cell lines, usually HEK293T cells and PT within the culture supernatant is harvested 48-72 hours later.

The PT particles are generated by the host cell machinery. The cell transcribes and translates the transfected genes and the reporter gene is incorporated into the PT core, , in the form of an RNA dimer. The glycoprotein is glycosylated and sent to the cell plasma membrane so that when the PT capsid arrives at the membrane it is able to bud out of the cell taking the plasma membrane expressing G with it. These enveloped PT virions now contain the reporter gene and the lentiviral reverse transcriptase and integrase proteins within the core which is surrounded by a plasma membrane from which the G protrudes. These mature virions are then capable of infecting cells

expressing G specific receptors. Because the

reporter construct is the only one to encode a packaging signal no other nucleic acids are present in the mature virion. This means that no viral proteins are generated in infected cells so no PT propagation occurs. This feature is the reason that PTs are such an effective vehicle to study lyssavirus neutralisation because they remove the requirement for working with live virus. This means that work can be done at a much lower containment level.

1.4 Reverse Genetics Techniques

The process of reverse genetics enables specific mutations to be engineered into viral genomes by a process of recovery of infectious virus from copy DNA (cDNA). Due to the different replication systems employed by DNA and RNA viruses, the process of reverse genetics was more readily applied to certain viruses than others. It was most quickly adapted to DNA viruses via heterologous recombination between plasmids containing specific viral sequences and the virus genome, or by directly transfecting cells with plasmids containing the required virus genome (Pekosz *et al.*, 1999). The next group of viruses to have successful reverse genetics systems were the positive strand RNA viruses. This is because the viral genome is mRNA sense. This meant that by transfecting cells with plasmids or RNA transcribed from plasmids which contain, for example, the poliovirus genome, infectious poliovirus was able to be generated (Racaniello & Baltimore, 1981). The negative strand RNA viruses however have several features which make artificial manipulation of their genomes more difficult than for other viruses: firstly specific 5’ and 3’ ends are required for the replication and packaging of genome RNA. Secondly the viral RNA polymerase is required to transcribe mRNA and positive sense antigenome RNA and finally, both genome and

antigenome RNA are incorporated into RNP complexes (Pekosz *et al.*, 1999). Another feature of negative sense RNA viruses which made development of reverse genetics systems more complex was the negative sense polarity of their genomes. These genomes are not able to act as a template to generate proteins; the viral RdRp must first transcribe the genome into positive sense. In addition, the transcription and replication of the genomes are dependent on sufficient quantities of cofactors being available, namely N protein to encapsidate the newly transcribed genomes. These features mean that particular systems have been developed in order to generate recombinant virus from cDNA, as shown in Figure 1.4.

This reverse genetic system has allowed a much more in depth understanding of the negative stranded viruses, including the lyssaviruses. Additional genes have been incorporated into viral genomes, to enable expression of both heterologous viral proteins and fluorescence markers. In addition, the function of individual genes or gene segments has been investigated via truncation and epitope mutation. Reverse genetics can also be applied to the development of novel vaccines and molecular tools (Finke & Conzelmann, 2005a). For the lyssaviruses, several recombinant approaches have been taken. A deletion mutant of RABV was generated that contained an alternative envelope alongside multiple fluorescent proteins produced transgenes enabling the resulting virus to efficiently infect and label neuronal axons at the site of infection (Wickersham *et al.*, 2013). Furthermore, attenuated RABVs have been generated as potential vaccine formulations. Alteration of P gene expression has been investigated by changing the position of P relative to the 3' genome terminus and by driving its expression from an internal ribosome delivery sites (IRES) (Marschalek *et al.*, 2009) (Brzozka *et al.*, 2005).

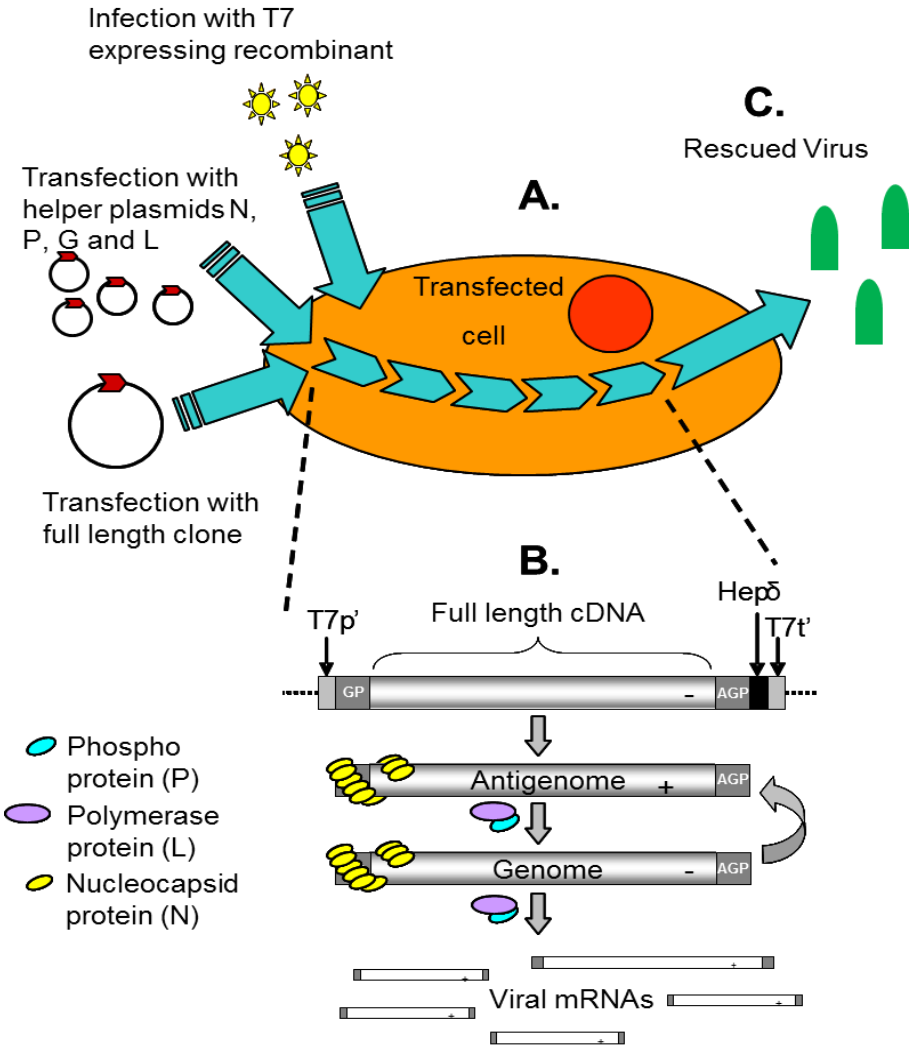


Figure 1.4: The process of reverse genetics rescue of non-segmented negative strand RNA viruses. A. Helper plasmids under the transcriptional control of the T7 polymerase, expressing the nucleoprotein (N), phosphoprotein (P), glycoprotein (G), large polymerase (L) and the cDNA clone are transfected into a cell which has previously been infected with a T7 polymerase expressing recombinant fowlpox virus. B. The antigenome (positive strand) sense T7 derived primary transcripts, are encapsidated by N protein and subsequently replicated to produce negative strand genomes; which are also encapsidated. The correct 3' end of the RNA is generated by the Hepatitis Delta (Hepδ) ribozyme sequence. These serve as the template for viral transcription and with the expression of the five viral proteins an infectious cycle begins. C. Rescued virus released from the cell. Adapted from (Conzelmann, 1996).

It has been shown that the pathogenicity of these viruses is markedly reduced in mice (Marschalek *et al.*, 2009; Rieder *et al.*, 2011) but their ability to protect against challenge has yet to be determined (Pfaller *et al.*, 2015). It is also possible for the glycoproteins to be switched between lyssaviruses so for example, a highly divergent glycoprotein can be incorporated into a vaccine strain in order to study neutralisation and glycoprotein function (Marston *et al.*, 2013).

1.5 Project Outline

The aim of this project is primarily to investigate the lyssavirus glycoprotein and its ability to induce neutralising antibodies. As the sole target of neutralising antibodies, and due to the inability of current vaccines to neutralise all lyssaviruses, mutations to G were investigated. The functionality, immunogenicity and neutralisation of different lyssavirus G constructs was assessed initially generating and using a panel of lyssavirus pseudotypes (PTs) representing different isolates from phylogroups I, II and III. The ability of PTs to be generated and the ability of standardised sera to neutralise these PTs was assessed using both wildtype and mutated glycoproteins as the target for neutralisation. PTs were also used to investigate the binding sites of mAbs proposed as potential alternatives to RIG. Following assessment of PT neutralisation, both wildtype and mutated G proteins were cloned into full length genomes and rescued using reverse genetics techniques. Both *in vitro* and *in vivo* assessment of virus growth and neutralisation was performed. Finally vaccination challenge experiments were conducted to evaluate the ability of the neutralisation profile following vaccination with the standard rabies vaccines to protect against challenge with recombinant viruses containing mutated G proteins.

Chapter 2: Materials and Methods

2.1 Cells and Viruses

2.1.1 Cell lines and maintenance

Baby hamster kidney cells (BHK-21), a fibroblastic cell line established from the kidneys of Syrian golden hamsters were grown in GMEM (Glasgow Modified Eagles Media) (Gibco) supplemented with 10% foetal bovine serum (FBS) (Gibco), 10% tryptose phosphate (Gibco), penicillin (100U/ml) and streptomycin (100µl/ml) (Gibco). Human embryonic kidney cells (HEK 293T/17), an endothelial cell line established from the kidney of a human foetus, were grown in DMEM (Dulbecco's Modified Eagles Media) (Gibco) supplemented with 10% FBS, 4g/L of D (+) glucose (Sigma Aldrich), penicillin (100U/ml) and streptomycin (100µl/ml). Tissue culture flasks (Sarstedt) were incubated at 37°C in a 5% CO_2 incubator (Binder).

2.1.2 Culturing cell lines

Maintenance media was removed from the cell culture flasks and cells were briefly washed with sterile Dulbecco's Phosphate Buffered Saline (DPBS) (Gibco). The DPBS was removed from the monolayer and a 4ml aliquot of 0.05% trypsin-EDTA (Gibco) was added to the cells. The flasks were incubated at 37°C for 2-5 minutes to detach the cells. Once detached, the cells were evenly suspended in 4ml of maintenance medium. Cells were either diluted by approximately 1:6 into flasks containing fresh medium for stock maintenance or they were counted using a haemocytometer (Improved Neubauer) then plated out for transfection, titration or pseudotype assay.

2.1.3 Freezing, storing and reviving cell lines

Cells were stored long term in liquid nitrogen (N_2). Preparation for long term storage was as follows: a flask of cells for freezing was trypsinised and the cell suspension was centrifuged at 500 rpm (34 x g) for 5 minutes. The cell pellet was resuspended in a 90% FBS, 10% Dimethyl Sulphoxide (DMSO) mixture at 1ml per 25cm^2 of cells. The suspension was then aliquoted into cryotubes (Nalgene) and placed overnight at -70°C in a Mr Frosty cooler (Thermo Scientific). Once frozen the tubes were transferred into liquid N_2 for storage. In order to revive the cells, tubes were thawed rapidly then gently suspended in the relevant medium. Cell suspensions were centrifuged at 500 rpm (34 x g) for 5 minutes and the resultant cell pellets were resuspended in 10ml of appropriate medium then transferred into a T25 tissue culture flask (Sarstedt).

2.2 Molecular biological techniques

Plasmid constructs were cloned and grown in *E. coli* (Max Efficiency DH5α cells (Invitrogen); JM109 competent cells (Promega); or TOP10 competent cells (Invitrogen). All bacterial preparations were cultured in Luria Bertani (LB) broth at 37°C.

2.2.1 Small scale plasmid preparations

Single colonies were picked from transformation plates and were grown in 2ml LB broth containing the appropriate antibiotic, usually either ampicillin or kanamycin at 100mg/ml (Sigma Aldrich) for 12-18 hours. A 1ml aliquot of each of these cultures was transferred to a fresh Eppendorf tube and the tubes were centrifuged at 14,000 rpm (21,000 x g) at 4°C for 30 seconds. The excess media was removed to leave the bacterial cell pellet. Next, 300µl of TENS buffer (10mM Tris/Cl, 1mM EDTA, 0.5% SDS and 0.1M NaOH) was

added to each tube and the samples were rigorously mixed by vortexing for 10 seconds. Then 150µl of 3M sodium acetate (NaOAc) (Sigma Aldrich) was added to each tube and the vortexing was repeated. The samples were then centrifuged for 3 minutes. After centrifugation the supernatants from each tube were transferred to fresh tubes containing 1ml of ice cold 100% ethanol. The samples were mixed by inversion then centrifuged for a further 3 minutes. DNA pellets were visible and the pellets were washed with 500µl of ice cold 70% ethanol by 2 minutes of centrifugation. The ethanol was then removed and the pellets left to air dry. The DNA was resuspended in 40µl of Tris-EDTA (TE) containing RNaseA (Qiagen) at a final concentration of 25µg/ml.

2.2.2 Large scale plasmid preparations

HiSpeed plasmid maxi kits (Qiagen) were used according to the manufacturer's instructions for large scale plasmid preparations (maxipreps). Briefly, plasmid DNA was re-transformed and cultured overnight in 150ml of LB broth containing the appropriate antibiotic. The cells were pelleted before being lysed in alkaline lysis buffer. Cell debris was removed by filtration and DNA was bound to the equilibrated Qiagen column. The column was then washed in wash buffer, followed by elution of DNA from the column and subsequent precipitation in isopropanol. The DNA was then filtered and stored in TE buffer at -20°C.

2.2.3 Restriction enzyme digests - preparative

Preparative digests were performed on different plasmid DNA preparations to prepare DNA for ligation. Restriction enzymes were used according to manufacturer's instructions (Promega, UK; New England Biolabs, USA). Up to 10µg DNA was cut in a final volume of 50µl.

2.2.4 Restriction enzyme digests – analytical

Analytical digests were performed on plasmid DNA preparations to determine the presence and orientation of ligated insert in a DNA sample. Restriction enzymes were used as per manufacturer's instructions (Promega, UK; New England Biolabs, USA). Between 400ng and 1µg of DNA was digested in a final volume of 10µl to 20µl.

2.2.5 Agarose gel electrophoresis

Agarose powder (Helena Biosciences Europe) was used to make 1-2% agarose gels in 1 x TAE buffer. 5µl of SYBR safe DNA gel stain (Invitrogen) was added per 100ml of cooled dissolved gel. Gels were left to set at room temperature for 30 minutes prior to loading. 6X loading dye (Promega) was used along with 1kb DNA ladder (Promega). Gels were run in 1 x TAE at 120V for 45 minutes.

2.2.6 Low melting point agarose gels

Bands resulting from preparative digests were separated by running digested DNA on low melting point (LMP) agarose gels (Invitrogen) of 0.8-2% to gel purify required DNA fragments. DNA bands were visualised under long wavelength UV light and the required bands were excised using a sterile scalpel. Excised DNA fragments were then purified from LMP gel using the Gfx purification kit (GE Healthcare) according to manufacturer's instructions.

2.2.7 Spectrophotometry

The concentration of DNA and RNA in solution was measured on a nanodrop ND-2000

machine (Thermo Scientific). Prior to all measurements a blank containing the diluting agent (TE or H_2O) was used to set the zero readings on the nanodrop machine. 1.2µl of each sample was measured on the nanodrop and the concentration and purity of nucleic acid was measured.

2.2.8 Cloning

2.2.8.1 Ligation reactions

Ligations were performed with 20-25ng of vector, with an insert:vector ratio of 1:1, 3:1 or 5:1, 1µl T4 DNA ligase (Thermo Fisher), 2µl 10x ligase buffer (Thermo Fisher) and water to a total of 20µl. Insert DNA, from PCR reactions or purified from gels was used in a range of ratios with 3:1 (insert to vector) being used most commonly and alternative ratios being used in cases of ligation failure. The ligation was incubated at 16°C overnight but in cases of ligation failure the repeat reaction was incubated on ice at room temperature overnight. Zero Blunt PCR cloning kit ligations using the pCR-Blunt vector (Invitrogen) ligations were performed according to manufacturer's instructions.

2.2.8.2 Gibson Assembly

An alternate approach to ligation of insert into vector using standard ligase was adopted to obtain certain constructs. Primers were designed to incorporate 19bp overlapping sequences containing the required restriction enzyme sequences. These were directed at the 5' and 3' ends of the vector and of the insert. A PCR reaction was carried out to obtain the overlapping insert and vector sequences. The reaction conditions used to obtain the insert sequences are detailed in section 2.2.10.1 with a variation in incubation, instead using a 57°C annealing temperature. The reaction to obtain the vector sequence comprised: 10µl

5X Q5 High-Fidelity DNA Polymerase buffer, 1µl 10mM dNTPs, 2.5µl forward primer, 2.5µl reverse primer, 1µl template DNA (vector plasmid diluted 1 in 100), 0.5µl Q5 High-Fidelity DNA Polymerase, 10µl Q5 high GC enhancer and 18.5µl water to make the reaction to 50µl. The reaction was carried using the following cycling conditions:

Step 1: 98°C for 30 seconds

Step 2: 98°C for 10 seconds

Step 3: 57°C for 30 seconds

Step 4: 72°C for 30 minutes

Step 5: repeat steps 2-4; 34 times

Step 6: 72°C for 30 minutes

Step 7: hold at 4°C

Once the overlapping PCR fragments had been obtained they were assembled using the following reaction: 2-10µl of the PCR fragments, 10µl of Gibson assembly 2X master mix and deionised water to 20µl. This was incubated at 50°C for 15 minutes then 2µl was used to transform NEB 5-alpha competent *E. coli*.

2.2.9 RNA extraction and reverse transcription

Total cellular RNA was extracted from infected cells using TRIzol reagent (Life Technologies) as per the manufacturer's instructions. 2µl of extracted RNA was used in reverse transcription reactions (RTs) to generate cDNA. Superscript III (Invitrogen) was used for all RT reactions following the manufacturer's instructions. Reactions were performed with random hexanucleotide primers (RHPs) (Invitrogen) or gene specific primers depending on the downstream requirement of the first strand cDNA synthesis reaction.

2.2.10 Polymerase chain reaction (PCR) techniques

2.2.10.1 KOD PCR

For the amplification of lyssavirus G open reading frames (ORFs) from both first strand RT reactions and plasmid DNA, KOD (Novagen) PCR was used. Each reaction used 1µl of cDNA (4-8ng/µl), 1.5µl of each primer at 10pmol/µl, 5µl of 10x buffer, 3µl of 25mM magnesium sulphate, 5µl dNTPs, 1µl of KOD polymerase and 32µl of water to make each reaction up to 50µl. The PCR was run on a PE2720/9700 thermocycler (Applied Biosystems) using the following cycling conditions:

Step 1: 95°C for 2 minutes

Step 2: 95°C for 20 seconds

Step 3: 60°C for 20 seconds

Step 4: 70°C for 20 seconds

Step 5: repeat steps 2-4; 34 times

Step 6: 70°C for 10 minutes

Step 7: hold at 4°C

The annealing temperature at step 3 was varied according to primer melting temperature (T_m).

2.2.10.2 Gradient PCR

Gradient PCR was used to optimise the generation of PCR products. An Agilent Technologies SureCycler 8800 was used to run KOD PCR reactions on temperature gradients of approximately 60°C ± 10°C across 6-12 tubes. The annealing temperature range was altered depending on primer T_m.

2.2.10.3 Overlap extension mutagenesis

To incorporate nucleotide mutations into antigenic sites within the G ORF, overlap extension mutagenesis was performed. Primers to incorporate the desired mutations were designed to overlap at the site of mutation by a minimum of 20 base pairs (bp). A first round KOD PCR was carried out using primers directed at the start and end of G to create a pair of fragments overlapping each region of mutation. The first round reaction cycling conditions were as follows:

Step 1: 95°C for 2 minutes

Step 2: 95°C for 20 seconds

Step 3: 60°C for 10 seconds (temperature varied depending on primer T_m)

Step 4: 70°C for 25 seconds

Step 5: repeat steps 2-4; 34 times

Step 6: 70°C for 10 minutes

Step 7: hold at 4°C

The pairs of fragments for each site were visualised by electrophoresis and if correct the second round KOD PCR reaction (see 2.2.10.1) to overlap the two fragments was carried out. The primers directed to the start and end of the G ORF were added to the reaction after 10 cycles to allow the overlapping fragments to anneal to each other prior to generating a complete amplicon.

2.2.11 Sequencing

Both PCR products and plasmid DNA were sequenced by the Central Sequencing Unit (CSU) at the AHVLA. Undiluted PCR product or plasmid DNA was sent to CSU along with primers at 1pmol/µl for PCR products or 0.8pmol/µl for plasmids. At CSU the

samples were cleaned and run on ABI 3130xl (16 capillary) and ABI 3730 (48 capillary) Genetic Analysers to obtain sequence reads.

2.2.11.1 DNASTAR Lasergene 9 core suite

The SeqMan program was used to align ABI Sanger sequencing produced sequence reads. The reads were aligned against an appropriate known glycoprotein sequence in order to confirm the sample's sequence.

2.2.11.2 Vector NTi Advance 11

Vector NTi (Invitrogen) was used to construct vector maps and for other routine molecular biology applications including restriction enzyme digestion prediction and sequence annotation.

2.2.11.3 Next Generation Sequencing on Illumina MiSeq Platform

Double stranded DNA from KOD PCR reactions was purified using AMPure XP magnetic beads (Beckman Coulter) and 1ng was used in the Nextera XT DNA sample preparation kit (Illumina). A sequencing library was prepared, according to manufacturer's instructions and this was sequenced on the Illumina MiSeq with 2 x 150 bp paired-end reads, according to standard Illumina protocols. The resulting reads were mapped to a reference sequence using the Burrows-Wheeler Aligner (WBA) (version 0.7.5a-r405) then were visualised in Tablet (Milne *et al.,* 2013). A modified SAMtools/vcfutils script (Li *et al.,* 2009) was used to generate an intermediate consensus in which any indels relative to the original reference sequence were appropriately called. The intermediate consensus sequence was used as the reference for subsequent iteration of mapping and consensus calling.

2.2.12 Construction of pseudotype plasmids

The backbone plasmid for pseudotype expression (pI.18- A kind gift from Dr Edward Wright, University of Westminster, London) contained a multiple cloning site flanked by the Cytomegalovirus immediate early (CMV IE) promoter and transcriptional terminators. Plasmid pI.18 was prepared to receive lyssavirus glycoproteins following digestion by restriction enzymes *KpnI* and *XhoI*. Each representative wildtype (wt) and recombinant lyssavirus G protein open reading frame was amplified using primers that incorporated *KpnI* and *XhoI* restriction enzyme sites for downstream cloning into pre-prepared pI.18. Blunt ended KOD polymerase generated lyssavirus G ORF PCR products were initially subcloned into pCR-Blunt (Invitrogen). Subclones were checked by restriction digestion to ensure insertion of the PCR product into the pCR-Blunt backbone plasmid. Correct subclone plasmids were then digested with *KpnI* and *XhoI* to release the lyssavirus G flanked by the correct restriction sites to enable directional cloning into the *KpnI* and *XhoI* prepared pI.18 plasmid. The insert was gel purified prior to ligation. Following ligation and transformation, small scale DNA preparations were made and successful ligation was checked using restriction enzyme digestion. Plasmids containing the correct insert were retransformed to enable generation of maxipreps.

2.2.13 SDS-PAGE analysis and Western blotting

Protein samples were generated by lysis of cells that had been transfected to produce pseudotype virus, using Reporter Lysis Buffer (Promega) and a freeze thaw cycle. The samples were then run on SDS-PAGE gels (BioRad) and were analysed by immunoblotting. The membranes (BioRad) were then blocked using 5% blotting grade buffer (BioRad) in TBS-0.2% (APHA, Reagent Production) Tween-20 (Fisher Scientific) overnight at 4°C. The primary antibody was added to 3ml blocking buffer and the

membrane was incubated in this mixture on a R100 Rotatest shaker (Luckham) at 4°C overnight. The membranes were then washed three times, each for 15 minutes, in TBS-0.2% Tween-20. The appropriate secondary antibody (polyclonal rabbit anti mouse immunoglobulins/HRP, Dako) was then diluted 1 in 1000 in 10mls of blocking buffer and the membrane was incubated in this antibody for 1 hour with constant mixing. The membranes were washed, as previously outlined, and the proteins were visualised using the ECL plus Western Blotting Detection System (Amersham) as per the manufacturer's protocol. The membranes were exposed to X-ray film (X-Omat LS, Kodak) and the results were visualised manually in a dark room using X-ray fixer and X-ray developer solutions (Champion Photochemistry).

2.2.14 Particle Tracking

A NanoSight LM10 machine (Malvern) was used to track pseudotype and virus particles in suspension according to the manufacturer's protocols. Briefly, pseudotype and virus preparations were diluted 1 in 100 in BHK media and 300µl was aseptically injected into the machine's specimen chamber until the liquid reached the nozzle tip. Specimens were tracked and measured for 60 seconds at room temperature. Data were captured and analysed using NanoSight LM10 NTA software (version 3.00).

2.3 Pseudotype techniques

2.3.1 Transient transfection

Transfections were carried out with Lipofectamine LTX reagent (Invitrogen) according to the manufacturer's instructions. Briefly, 0.72µg of glycoprotein, 0.72µg of pGagPol and 1.08µg of reporter plasmid were added per well of a 6 well plate. Transfection reagent was

optimised for the procedure with a Lipofectamine LTX:DNA ratio of approximately 6:1 being determined as being optimal for reporter gene expression. The cells were incubated at 37°C and 5% CO_2 for 48 hours before harvesting the pseudotype virus.

2.3.2 Pseudotype titration

The titres of harvested PT viruses were determined by 50% tissue culture infective dose ($TCID_{50}$). Each PT was titrated in triplicate in a 96 well flat bottomed tissue culture plate (Thermo Fisher). 100µl of BHK media was used per well required and 6 serial 5-fold or 10-fold dilutions of PT stock were made in BHK media. 50µl of PT was added per well in triplicate down the plate. BHK cells were split and diluted to $2x10^5$ cells/ml then 50µl was aliquotted per well. The bottom row was left as a cells alone/uninfected control. The plates were incubated for 48 hours then the media was removed and replaced with 50µl of a mixture of Bright-Glo reagent (Promega) and BHK media in a 1:1 ratio. Luciferase activity was measured 2.5 minutes later on a Glomax 96 microplate luminometer (Promega). The negative cut off value was defined as the average cells alone luminescence multiplied by 2.5. The last dilution at which all wells for each PT were positive was used to determine the PT titre in $TCID_{50}$/ml via the Spearman-Kärber method (Aubert, 1996).

2.3.3 Pseudotype neutralisation assay (PNA)

The pseudotype assay was adapted from the procedure previously described (Wright *et al.*, 2008). In order to determine the percentage neutralisation of a PT by a specific serum the pseudotype virus was diluted to 200 $TCID_{50}$/50µl. It was then incubated with 50µl of sera at 0.5 IU/ml or a range of serum concentrations for 1 hour at 37°C in 5% CO_2. In order to determine the virus neutralising titre of a serum sample the serum was diluted 1:20 in

duplicate in the top two rows of a 96 well plate. Twelve serial 2-fold dilutions of the serum were then made down the plate and 50µl of PT at 200 $TCID_{50}$ was added to each well. The plates were then incubated for 1 hour at 37°C in 5% CO_2. In both instances after incubation, 50µl of cells from a suspension of $2x10^5$ cells/ml were added per well. The plates were incubated for a further 48 hours then the media was removed and replaced with 50µl of a mixture of Bright-Glo reagent (Promega) and BHK media in a 1:1 ratio. Luciferase activity was measured 2.5 minutes later on a Glomax 96 microplate luminometer (Promega). For the percentage neutralisation test, the degree of neutralisation was calculated as a reduction in the level of luminescence compared to virus not exposed to serum. Each assay was carried out in triplicate and a mean average percentage neutralisation was calculated. For the serum titre test the 50% end point dilution (where complete neutralisation stopped in 50% of the wells) was calculated via the Spearman-Kärber method (Aubert, 1996).

2.4 Manipulation of full length clones to insert heterologous lyssavirus glycoproteins

The backbone plasmid for full length expression (cSN) contains the full genome for a phylogroup I classic rabies virus. cSN was prepared for ligation of lyssavirus glycoproteins (G) by restriction enzyme digestion with *SmaI* and *NheI* (Promega) to excise the backbone phylogroup I G. Each representative wildtype and recombinant lyssavirus G protein open reading frame (ORF) was amplified using primers that incorporated *HpaI* and *NheI* (Promega) restriction enzyme sites for downstream cloning into pre-prepared cSN. Blunt ended KOD polymerase (Novagen) generated lyssavirus G ORF PCR products were initially subcloned into pCR-Blunt (Invitrogen). Subclones were checked by restriction

digestion to ensure insertion of the PCR product into the pCR-Blunt backbone plasmid. Correct subclone plasmids were then digested with *HpaI* and *NheI* to release the lyssavirus G flanked by the correct restriction sites to enable directional cloning into the *SmaI* and *NheI* prepared cSN plasmid. Following ligation and transformation, small scale DNA preparations were made and successful ligation was checked using restriction enzyme digestion. Plasmids containing the correct insert were retransformed to enable generation of maxipreps.

2.5 Virus rescue

Rescue of full length clones of CVS and LBV antigenic site swaps and wild type viruses were carried out in BHK cells using FuGENE 6 (Promega). Cells were plated at $5.4x10^5$ cells per well to give 60-70% confluence. Each well was infected with fowlpox T7 (FPT7) to provide the T7 RNA polymerase. After one hour of incubation at 37°C the FPT7 was removed and each well was transfected with 1µg of pN, 1µg of pP, 1µg of pL and 2µg of genome plasmid all under the transcriptional control of the T7 polymerase. Plates were incubated for 48-72 hours prior to harvest.

2.5.1 Virus Titration

The titres of harvested viruses were determined in focus forming units per ml (ffu/ml). Each virus was titrated in triplicate in a 96 well flat bottomed tissue culture plate (Thermo Fisher). 100µl of BHK media was used per well required and 10µl of virus was added in triplicate along row A of the plate. Eight serial 10-fold dilutions of virus were made down the plate to row H. BHK cells were split and diluted to $5x10^5$ cells/ml then 50µl was added to every well. The plates were incubated for 48 hours then the media was removed and replaced with 50µl of 80% acetone (Fisher Chemicals). After 20 minutes fixation at room

temperature the acetone fixative was removed and the wells left to completely air dry. Once all wells were dry 50µl of a 1:100 dilution of anti-N FITC conjugated mAb (Fujirebio) was added to every well and the plates incubated at 37°C for 30 minutes. After the incubation the mAb was removed, the cells were washed twice with sterile 0.1M PBS and the plates were visualised using a fluorescence microscope to detect cell specific apple green perinuclear fluorescence which indicated presence of N protein. The number of foci in each well was recorded and the average number of foci in the last row before no foci were present was used to determine the ffu/ml of each virus.

2.6 Fluorescent Antibody Virus Neutralisation (FAVN) test

The neutralising ability of sera was assessed using the FAVN test against CVS virus. Serial 3-fold dilutions of serum were made in BHK media in a 96 well plate (Corning) using four rows per serum. Stock CVS virus was then diluted to 100 $TCID_{50}$/50µl and 50µl was added to every well. The plates were incubated at 37°C at 5% CO_2 for one hour. After incubation 50µl of a suspension of 5 x 10^5 BHK cells/ml was added to every well and the plates were incubated for a further 48 hours. After incubation the supernatant was discarded and the cells were fixed with 80% acetone (Fisher Chemicals) for 20 minutes at room temperature. The monolayers were air dried then stained with 50µl per well of a 1:100 dilution of murine anti-N mAb conjugated to fluorescein isothiocyanate (FITC) (Fujirebio Diagnostics Inc.). The wells were then washed twice with 0.1M PBS and visualised using a fluorescence microscope. The 50% endpoint dilution (where complete neutralisation stopped in 50% of wells) was calculated using the Spearman-Kärber method (Aubert, 1996).

2.7 Immunofluorescence techniques

2.7.1 Pseudotype visualisation

Cells grown on 16mm glass coverslips (VWR) that had been either transfected or infected were washed three times with 0.1M PBS. The cells were then fixed in 4% paraformaldehyde (PFA) (Affymetrix) for 30 minutes. After the cells had been fixed they were permeabilised with 0.1% Triton X100 (Sigma-Aldrich) in PBS for 10 minutes. The cells were then washed with 0.5% BSA (Sigma-Aldrich) in PBS (Gibco) to block non-specific binding. The PBS-BSA was aspirated and replaced with an anti-G protein murine primary antibody (MyBioSource) diluted 1:50 in PBS-BSA then incubated for 30 minutes. The cells were washed three times in PBS-BSA then a goat anti-mouse 568-conjugated secondary antibody (red) (Life Technologies) at a 1:200 dilution was added for 20 minutes at room temperature. This was washed off with three PBS-BSA washes. The cells were then stained with an actin-phalloidin 488-conjugated mAb (Invitrogen) diluted 1 in 50 in PBS-BSA for 20 minutes. Following three PBS-BSA washes the coverslips were then mounted on glass microscope slides (VWR), cell side down, in aqueous mounting solution containing 4′,6-diamidino-2-phenylindole (DAPI) DNA stain (Vectashield, Sigma-Aldrich). The coverslips were sealed with nail varnish then visualised on a Leica SP8 confocal scanning laser microscope.

2.7.2 Recombinant virus visualisation

BHK cells were infected with rescued recombinant lyssaviruses. Following an incubation period of approximately 48 hours, infected cells were fixed in 4% PFA (Affymetrix) for 30 minutes at room temperature then permeabilised with 0.1% Triton X-100 (Sigma-Aldrich) for 10 minutes also at room temperature. The cells were then stained to identify

nucleocapsid aggregates suggestive of lyssavirus presence in order to confirm the success of the rescue. Murine anti-N mAb conjugated to fluorescein isothiocyanate (FITC) (Fujirebio Diagnostics Inc.) was used at a 1/100 dilution to cover the cell monolayer. The plate was then incubated at 37°C with 5% CO_2 for 30 minutes. The cells were then washed twice with 0.1M PBS and visualised on a Leica L5 microscope in the blue excitation wavelength between 475-490 nm. The presence of distinct bright 'apple' green fluorescence, usually in the perinuclear area of cells confirmed presence of lyssavirus. After the N protein had been stained an anti-G protein murine primary antibody (MyBioSource) was added at a 1:50 dilution for 30 minutes at room temperature. The cells were washed three times in 0.5% BSA (Sigma-Aldrich) in PBS (Gibco) followed by the addition of a goat anti-mouse 568 secondary antibody (Life Technologies) at a 1:200 dilution for 20 minutes at room temperature. This was then removed and the cells were washed thrice in PBS-BSA. The coverslips were then removed from the wells, gently blotted dry and mounted on glass microscope slides (VWR) in 5µl aqueous mounting solution containing 4′,6-diamidino-2-phenylindole (DAPI) DNA stain (Vectashield, Sigma-Aldrich). The coverslips were sealed with nail varnish then visualised on a Leica SP8 confocal scanning laser microscope.

2.8 Flow Cytometry

HEK 293Tcells were transfected to produce PT following the procedure in Section 2.3.1. Supernatants were harvested and the remaining cell monolayer was resuspended in DPBS supplemented with 2% FBS. The cells and the supernatants were stained with an anti-rabies glycoprotein mAb (MyBioSource) at a dilution of 1/50 for 30 minutes at 4°C. The primary antibody was then washed off and replaced with a 568-conjugated goat anti-mouse secondary antibody (Invitrogen) at a dilution of 1 in 200. The cells and supernatants were

stained in the secondary antibody at 4°C for 30 minutes. The cells and PTs were washed and resuspended in 2% FBS DPBS then analysed on a MACSQuant flow cytometer (Miltenyi Biotec).

2.9 *In vivo* studies

2.9.1 Rabbit Inoculation with PT

2.9.1.1 Production of Inocula

A panel of lyssavirus PTs were generated using the procedures detailed in Section 2.3. These were then beta-propiolactone inactivated. This involved the addition of beta-propiolactone at a 1/4000 dilution of the final volume of PT. These were incubated at 4°C for approximately 19 hours followed by 1 hour at 37°C. A neutral pH of the inactivated PTs was indicated by an orange colour of the media. These inactivated preparations were then separated into one aliquot per inoculation and stored at -20°C until reconstitution with adjuvant. The adjuvant used in this experiment was Montanide™ ISA 50 V2 (Seppic) at a 1:1 dilution with inactivated PT. Prior to each inoculation one vial of each inactivated PT was thawed and combined with an equal volume of Montanide™ to form an emulsion. These were immediately inoculated to avoid sedimentation.

2.9.1.2 Inoculation of Rabbits

One rabbit per PT, i.e. 10 1 year old female rabbits, were housed together in conditions consistent with Home Office regulations for a period of 10 days after arrival onsite at APHA and prior to the initial inoculation. Food and water were provided *ad libitum* along with sufficient environmental enrichment. Before inoculation, sites for subcutaneous inoculation were shaven and Emla cream (AstraZeneca) was generously applied to these

areas for a period of up to 5 minutes. The inocula were then injected at four sites subcutaneously followed by two sites intramuscularly at days 0, 21, 28 and 35. On day 43 all rabbits were anaesthetised, a maximal volume of blood was removed via cardiac puncture under terminal anaesthesia.

2.9.1.3 Collection of Sera from Rabbits

After the blood had been collected from each rabbit this was subjected to centrifugation at 2500 rpm (860 x g) for 10 minutes, after which the serum was removed from the blood pellet. The sera were then heat inactivated at 56°C for 30 minutes then separated into small aliquots and stored at -20°C until required.

2.9.2 Vaccination/Challenge in Mice

2.9.2.1 Mice

Three to four week old CD1 mice (Charles River) were housed in animal containment facilities compliant with current Home Office requirements (PPL 70/7394) with access to food and water ad *libitum* along with appropriate environmental enrichment. Each mouse was microchipped using Trovan chips to enable identification.

2.9.2.2 Vaccination of mice

The human rabies vaccine Rabipur (Novartis) was reconstituted as per the manufacturer's instructions in 1ml of sterile water immediately prior to vaccination of the mice. The reconstituted vaccine was then diluted 1 in 20 in sterile filtered deionised water. Mice were anaesthetised using isofluorane and vaccine was introduced via intraperitoneal injection of 500µl to the lower right hand quadrant of the abdomen. Mice were vaccinated on days 0

and 14 to ensure a high serological response to vaccination.

2.9.2.4 Blood Sampling by Tail Bleed

Twenty one days post vaccination a sample of blood was taken from each mouse in order to confirm seroconversion following vaccination. The dorsal tail vein of each mouse was nicked under anaesthesia using a scalpel blade and the resulting blood was collected in CB 300 tubes (Sarstedt). Following collection, blood samples were stored at 4°C overnight prior to centrifugation at 2500 rpm (860 x g) for 10 minutes after which the serum was separated from the blood pellet. These serum samples were heat inactivated at 56°C for 30 minutes and stored at -20°C until required for virus neutralising antibody detection.

2.9.2.5 Intracranial Challenge with Virus

Once virus neutralising antibody titre had been determined mice were challenged intracranially with 100 ffu/30µl of infectious recombinant viruses at 28 days post vaccination. Each challenge group was inoculated with 30µl neat virus directly through the cranium into the brain. The mice were housed in groups of 5 per cage and observed twice daily for signs of clinical disease. Clinical scores were recorded for each mouse according to the clinical signs scale (Appendix III). Any mice showing signs of clinical disease of score 3 were immediately anaesthetised, blood collected by cardiac puncture (Section 2.9.2.6) and subsequently euthanised by cervical dislocation.

2.9.2.6 Blood Collection via Cardiac Puncture

Mice that showed signs of clinical disease of score 3 and those which survived until the termination of the experiment were anaesthetised with isofluorane. Blood was drained

directly from the heart by introducing a needle into the chest cavity and collecting the blood in a syringe. This was followed by immediate cervical dislocation. The blood was then processed to isolate the sera as described for tail bleeding in Section 2.9.2.4.

2.9.3 Pathogenicity Study

2.9.3.1 Mice

Three to four week old CD1 mice (Charles River) were housed in animal containment facilities compliant with current Home Office requirements (PPL 70/7394) with access to food and water ad *libitum* along with appropriate environmental enrichment. Each mouse was microchipped using Trovan chips to enable identification.

2.9.3.2 Footpad Inoculation with Virus

After acclimatisation to the environment for 5 days prior to inoculation the mice were inoculated into the footpad with 1000 ffu/50µl of each virus, except cSN-LBVFSS which had a low titre so was inoculated neat; at 200 ffu/50µl. Prior to inoculation the mice were anaesthetised with gaseous isofluorane. Once the mice were sufficiently anaesthetised they were placed into a holding container with a small hole, through which the left hind limb was extended to enable inoculation. Mice were monitored until 28 days post infection. Clinical signs were scored and recorded using the clinical scoring system (Appendix III). Mice were euthanised via cardiac terminal bleed and cervical dislocation at clinical score 3 (Section 2.9.2.6).

Chapter 3: Construction and characterisation of wildtype and mutant forms of lyssavirus pseudotypes

3.1 Introduction

The adaptation of pseudotype viruses to express lyssavirus glycoproteins has several advantages for utilisation as research tools. Firstly, as previously described, the application of PTs to serological work enables the performance of neutralisation assays outside of containment facilities. This is of great benefit when working with ACDP3/SAPO4 pathogens and the diagnostic application of this technology for rabies serology is currently being trialled in resource limited settings. Secondly, the assay requires less serum than is required for OIE recommended tests and so is advantageous when assessing the serological status of species where only low volumes of sera are available, for example bats. Finally, it enables the mutation of the glycoprotein (G) alone to assess the effect of the antigenic regions on the ability of different sera to neutralise different wildtype and mutant glycoproteins. It is this final feature that is the basis for the present chapter.

Plasmids containing both wildtype and mutant G genes were constructed to enable the generation of lyssavirus pseudotypes representative of the genus as well as mutants containing swaps in the highly immunogenic epitopes described in Section 1.1.2.4.1. The discovery of two novel lyssaviruses during this study, Bokeloh Bat Lyssavirus (BBLV) and Ikoma lyssavirus (IKOV) also prompted the cloning and generation of G plasmids for these two novel viruses. Alongside plasmid construction and transfection to generate PTs, the optimisation of transfection conditions is described with data demonstrating the variability in titres obtained with different plasmids.

3.2 Generation of wildtype lyssavirus pseudotype plasmids

Numerous lyssavirus pseudotype plasmids were available at the initiation of the project through collaboration with Dr Edward Wright (University College London/University of Westminster). However, the discovery of two novel lyssaviruses during the project meant that pseudotype plasmids were required that contained the open reading frames (ORF) for BBLV and IKOV G. To this end, PCR amplicons were generated by overlap PCR for both the BBLV and IKOV G ORFs using KOD polymerase. Restriction sites (*KpnI* and *XhoI*) were incorporated at the 5' and 3' end of each ORF through inclusion within primer sequences (Appendix II). Both direct digestion of PCR products and blunt ended cloning of KOD generated PCR amplicons was performed. Where attempts to clone directly digested PCR products failed, G ORFs were excised from blunt end cloned plasmids and gel purified before being ligated into prepared vector. Following transformation into *E. coli.*, colonies were screened by restriction enzyme digestion and clones giving the correct digestion pattern were sequenced to confirm the insert sequence. The cloning strategy for the generation of pseudotype expression plasmids is detailed in Figure 3.1.

3.3 Construction of single site antigenic mutant PT plasmids

Antigenic site swap CVS mutants containing single LBV NIG 1965 (lineage B) sites were constructed along with the reciprocal swap of LBV to CVS. These mutants were generated by overlap extension mutagenesis and site directed mutagenesis and kindly donated by Dr. E. Wright at the University of Westminster. These mutants constitute swaps between phylogroup I and phylogroup II and can be seen in Figure 3.2.

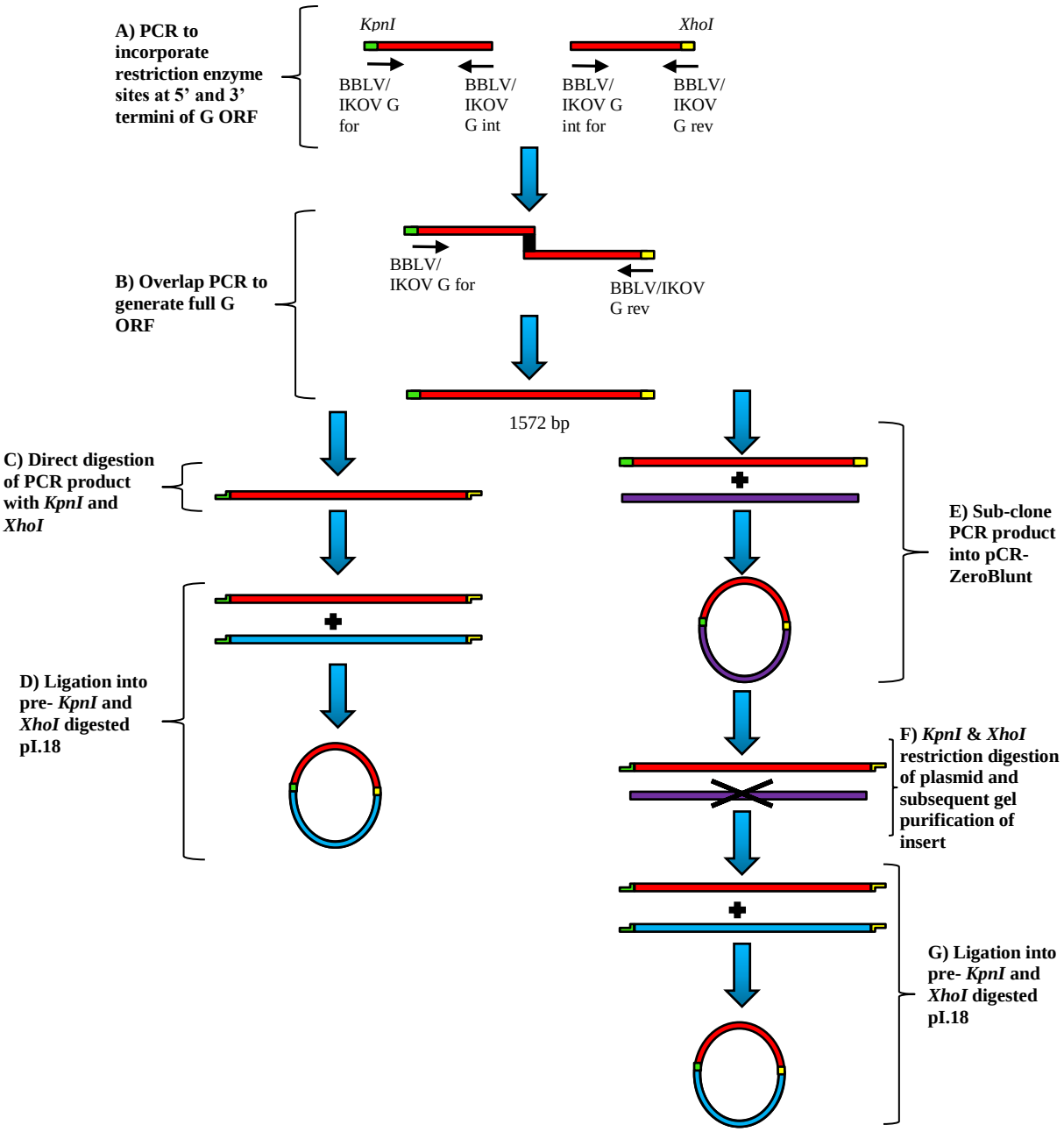


Figure 3.1: The cloning strategy to generate PT expression vectors for BBLV and IKOV. Insert DNA is highlighted in red, the pCR-ZeroBlunt vector DNA is in purple and the pI.18 vector DNA is in blue. The restriction sites *KpnI* and *XhoI* are in green and yellow, respectively.

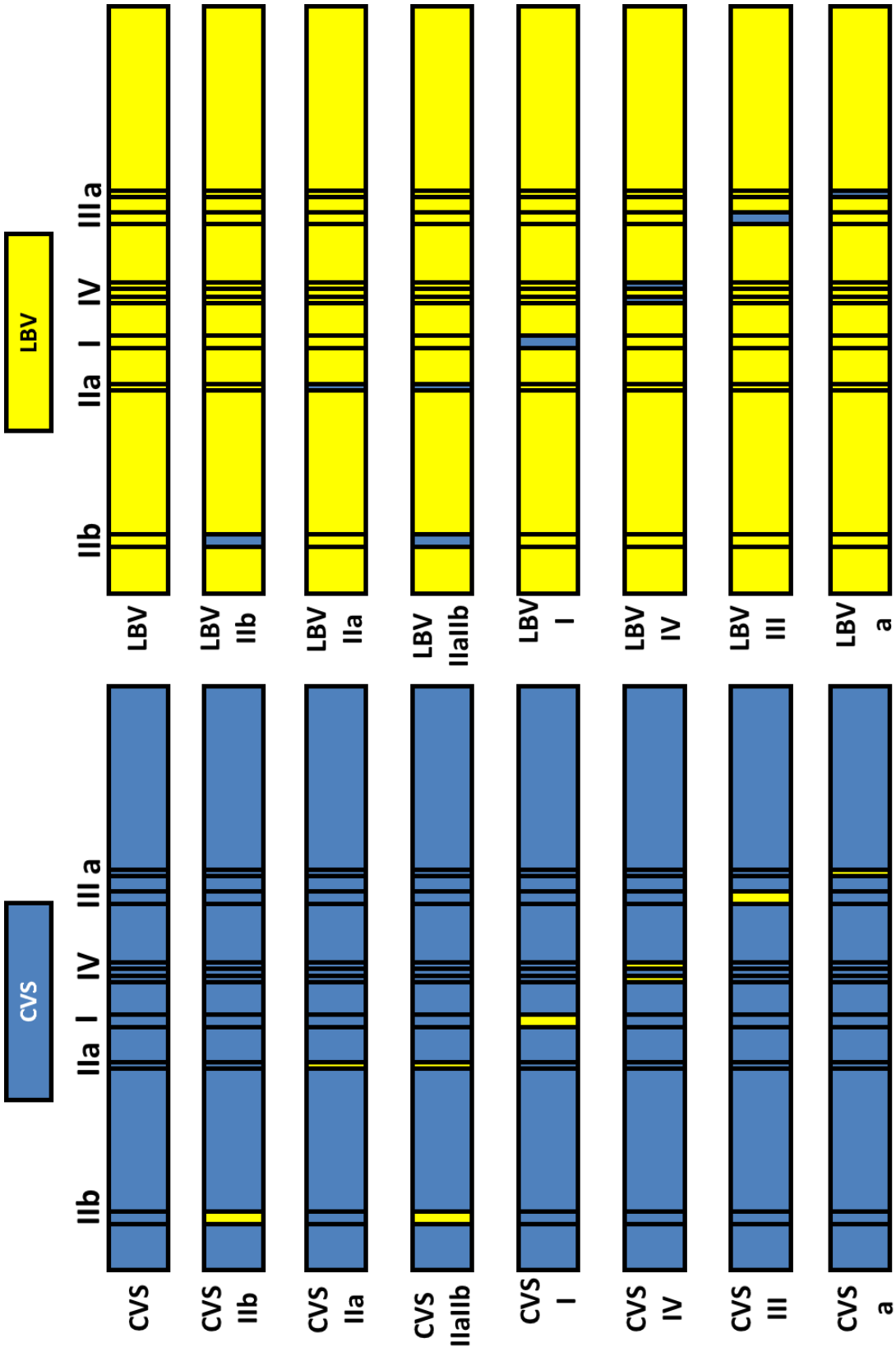


Figure 3.2: Schematic representations of each of the CVS and LBV antigenic site swap mutant glycoproteins. Where regions contain sequence from CVS they are coloured blue whilst sequences from LBV are coloured yellow.

3.4 Synthesis and cloning of CVS/LBV full site swap antigenic mutants

Genes containing a full complement of mutated antigenic sites were synthesised commercially as a cost effective alternative to individual site directed mutagenesis across the G ORF. Two genes were synthesised: 1) the CVS G gene containing the antigenic sites swapped with those of LBV lineage B and; 2) the LBV lineage B gene containing the antigenic epitopes swapped with those of CVS. Both synthesised genes incorporated the *KpnI* and *XhoI* sites at the 5' and 3' termini, respectively. The G protein DNA was then excised from the synthesised vector with a *KpnI* and *XhoI* restriction enzyme digest and the resulting fragment was purified and ligated into pI.18. Small scale plasmid preparations were made of selected colonies and restriction enzyme mapping was utilised to confirm the correct insertion sequence. Two correct small scale DNA preparations were then selected for re-transformation and subsequent large scale DNA preparation. Sequencing of PCR products of the G ORF from these plasmids were used to confirm the G sequence, prior to the use of these in transfection.

3.5 Optimisation of transfection for PT production

Previous studies had indicated that pseudotype production requires optimisation in each laboratory setting (Dr E. Wright, University College London/University of Westminster-personal communication) and as such optimisation of the assay was performed to maximise the production of high titre lyssavirus pseudotypes

Two transfection reagents were available in the laboratory; FuGENE 6 (Promega) and Lipofectamine LTX (Life Technologies) so these two reagents were compared in order to identify which produced the highest PT titres.

The manufacturer's recommended amount of each transfection reagent along with increasing quantities were added into transfections to produce the CVS PT. Three volumes of FuGENE 6: 6, 12 and 18µl and four volumes of Lipofectamine LTX were compared: 6, 9, 12 and 15µl. The rest of the protocol followed the manufacturer's instructions. The resulting CVS PTs were titrated on a 5 fold dilution series and the $TCID_{50}$/ml titres were calculated. The results are displayed in Figure 3.3.

The Lipofectamine LTX transfections show a positive correlation between titre and volume of transfection reagent used. They do however show a greater level of variation at each titre than the FuGENE 6 values. The titres with FuGENE 6 show that the lowest PT titre was obtained with 12µl of transfection reagent and this was the lowest titre of all obtained ($10^{3.05}$ $TCID_{50}$/ml). The highest titre using the lowest volume of transfection reagent, 6µl, was with FuGENE 6 at $10^{3.63}$ $TCID_{50}$/ml; however the titres with FuGENE 6 decreased after this point, unlike with Lipofectamine LTX. The highest titre obtained overall was $10^{4.13}$ TCID/ml, using 15µl of Lipofectamine LTX and for this reason, this reagent at a volume of 15µl was chosen for use in all subsequent transfections to generate a panel of PT viruses.

3.6 Production of a panel of lyssavirus PTs

Once the process of generating pseudotype viruses had been optimised and was reliably able to produce PTs with viable titres, work began on production of a range of PT viruses. The panel required included a full complement of wildtype, single antigenic site swaps (SSS) and full antigenic site swaps (FSS) between CVS and LBV.

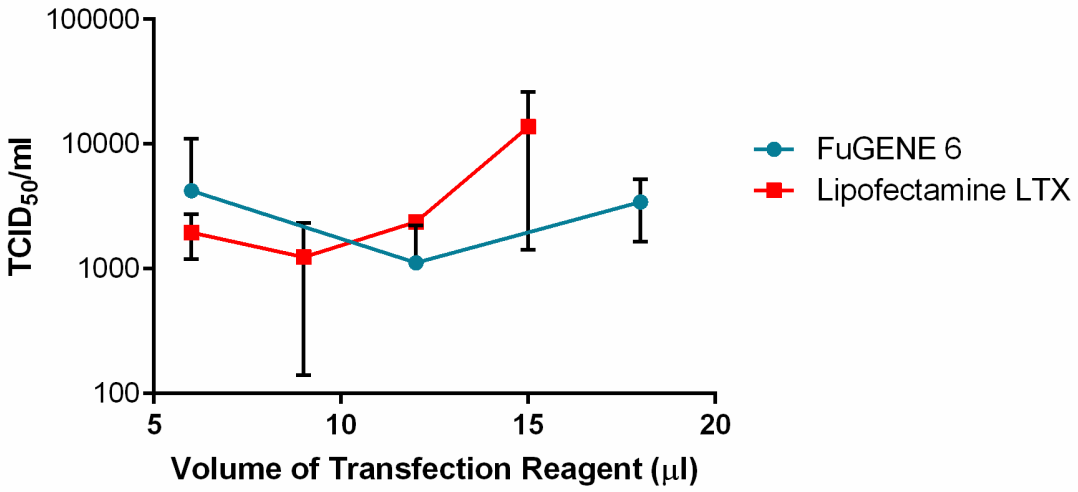


Figure 3.3: Optimisation of reaction conditions for the production of viral pseudotypes.

Comparison of CVS PT generated by increasing amounts of either FuGENE 6 or Lipofectamine LTX transfection agents. In the FuGENE 6 transfections 0.6ng both pGagPol and pGlycoprotein along with 0.9ng pReporter plasmid were transfected. In the Lipofectamine LTX transfections 0.72ng both pGagPol and pGlycoprotein along with 1.08ng pReporter plasmid were transfected. Error bars represent standard deviation (SD) about the mean of three replicate in-assay titrations.

It also included generation of PTs for a wide range of other wildtype lyssaviruses including a selection of RABVs, representatives of all other phylogroup I viruses, single isolates of all four lineages of LBV alongside other phylogroup II viruses as well as WCBV from phylogroup III and the two novel lyssaviruses, BBLV and IKOV. The harvested PTs were titrated via a 5-fold or 10-fold dilution series to obtain a $TCID_{50}$ titre, as shown in Figure 3.4.

The titres of the phylogroup I PTs range between 10^3 and 10^6 $TCID_{50}$/ml with CVS and DUVV having the highest titres, closely followed by EBLV-2. The rest of the PT titres were all very similar at approximately $10^{3.7}$ $TCID_{50}$/ml, with KHUV having the lowest titre at 10^3 $TCID_{50}$/ml.

The titres of the phylogroup II PTs show some variability with LBV A and WCBV having the highest titres at $10^{6.29}$ and $10^{6.54}$ $TCID_{50}$/ml, respectively. LBV B, MOKV and SHIBV all have similar titres between 10^4 and 10^6 $TCID_{50}$/ml but LBV D shows a titre of only $10^{1.99}$ $TCID_{50}$/ml. Due to the possibility of variation between individual plasmid preparations an alternative LBV D plasmid preparation was generated and the transfection was repeated in order to try and improve this PT titre. The PT resulting from this repeated transfection and subsequent titration, however remained at the same titre of $10^{1.99}$ $TCID_{50}$/ml which suggested that a feature of the glycoprotein itself may be affecting the titre achieved.

The titres of the CVS and LBV PTs varied considerably, regardless of the presence of antigenic site mutations. The CVS site swap PT titres were more variable than the LBV site swap titres. The LBV PT titres all showed similar titres with most reaching

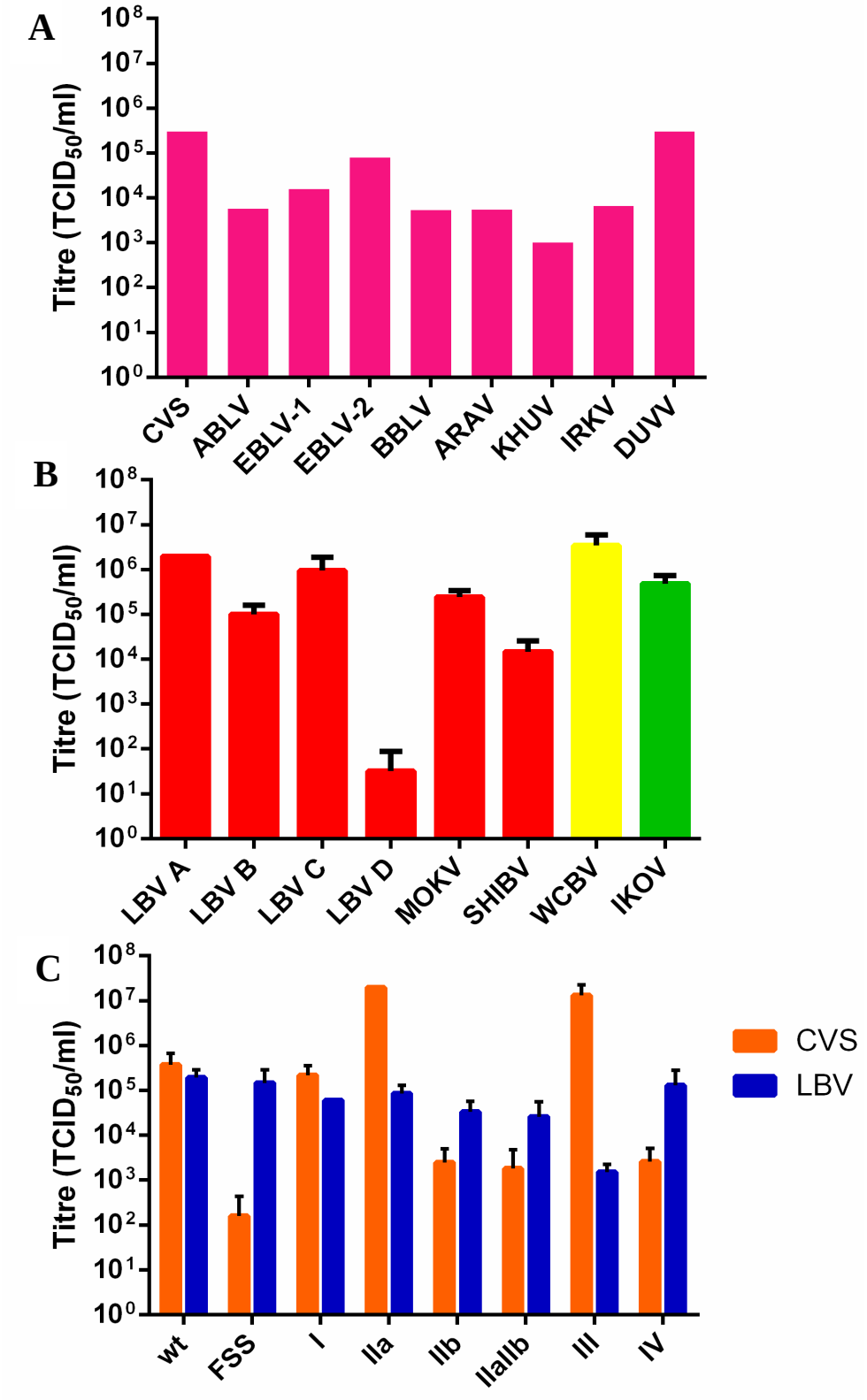


Figure 3.4: Pseudotype titres achieved for a representative panel of lyssaviruses. (A) PT titres of representatives of all lyssaviruses in phylogroup I. (B) The titres of representatives of phylogroup II in red, phylogroup III in yellow and IKOV in green. (C) The titres of all of the CVS and LBV antigenic site swap and wildtype PTs. Error bars represent standard deviation (SD) about the mean of three replicate in-assay titrations.

approximately 10^5 $TCID_{50}$/ml regardless of the antigenic site mutation present except LBV to CVS site III which had a lower titre of $10^{3.18}$ $TCID_{50}$/ml. In contrast, for the CVS PTs some of the antigenic site mutations appeared to have affected the final titres achieved. The CVS wildtype and site I PTs showed titres similar to their LBV counterparts whereas the CVS site IIa and III PTs had titres dramatically higher than the equivalent LBV PTs.

In addition, the CVS to LBV site IV PT reached a considerably lower titre than its reciprocal LBV counterpart confirming initial findings with this plasmid (Dr Ed Wright – unpublished data and personal communication). The CVSFSS PT also had a very low titre and for this reason a number of attempts were made to increase the titre of this PT (see Table 3.1). Both FuGENE 6 and Lipofectamine LTX were utilised to determine which produced higher titres, in addition an alternative cell type was used for transfection; BHKs, but no viable PT was generated and so repeated attempts were made with the HEK 293T cells. An alternative plasmid preparation with a higher concentration was produced and used in transfection. Transfection of the new construct enabled the production of PT, albeit at a very low level. In order to attempt to increase the titre increasing amounts of G plasmid DNA were transfected, however, none of these alterations were able to produce a viable titre. This suggested that the CVSFSS glycoprotein could not be pseudotyped.

Comparing the titres of the single antigenic site swaps of the CVS PTs (Figure 3.4) showed that the CVS IIb and IIaIIb PTs also only achieved low titres of $10^{1.79}$ and $10^{2.02}$ $TCID_{50}$/ml, respectively. This suggested that the alteration of the residues that are present within the IIb antigenic site may be affecting titre. In an attempt to increase the

Table 3.1: Alterations to transfection procedures to attempt successful generation of a CVSFSS PT.

Attempt	Cell type transfected	Transfection reagent	Concentration of Envelope plasmid (µg/µl)	Quantity of Envelope pDNA transfected (µg)	Titre ($TCID_{50}$/ml)
1	HEK293T-17	FuGENE 6	0.36	0.6	$10^{1.99}$
2	HEK293T-17	FuGENE 6	0.36	0.6	$10^{1.65}$
3	BHK	FuGENE 6	0.36	0.6	0
5	HEK293T-17	Lipofectamine LTX	1.00	0.72	$10^{2.20}$
6	HEK293T-17	Lipofectamine LTX	1.00	1.1	0
7	HEK293T-17	Lipofectamine LTX	1.00	1.4	0
8	HEK293T-17	Lipofectamine LTX	1.00	1.8	0

CVSFSS titres, reversion of the LBV IIb site mutation back to wildtype CVS was made in the CVSFSS constructs using overlap extension mutagenesis. The reversion of LBV site II back to CVS in the CVSFSS construct to produce two novel constructs: CVSFSS to CVS IIb and CVSFSS to CVS IIaIIb was not successful in rescuing PT titre. In fact the titres of the reversion mutants are slightly lower than the original CVSFSS titre of $10^{2.20}$ $TCID_{50}$/ml. The titres of the resulting PTs can be seen in Figure 3.5.

In an additional attempt to increase the titre of CVSFSS along with the low titre LBV D PT an alternative transfection strategy was adopted which involved a number of alterations to the protocol in Section 2.3.1. Initially HEK293T-17 cells were seeded into 6 well plates at a lower count than the previous protocol; this time at 5 x 10^5 cells/ml and were grown in OptiMEM (Gibco). The following day the CVSFSS or LBV D G plasmid alone were transfected into the cells, using the same quantity of G plasmid and same ratio of DNA:transfection reagent as before. Then 48 hours post transfection, the media was replaced with fresh OptiMEM and a transfection identical to that in Section 2.3.1 was performed, but now, the cells were maintained in OptiMEM for a further 48 hours post transfection and after this the tissue culture supernatant was collected and the PT within was titred as in Section 2.3.2. The titre of the CVSFSS PT did not increase as a result of this double transfection, it remained low at $10^{1.65}$ $TCID_{50}$/ml. The LBV D PT however did increase in titre from $10^{1.99}$ to $10^{2.34}$ $TCID_{50}$/ml. This LBV D titre remains fairly low in comparison to other wt PT titres and the CVSFSS titre did not increase at all which confirms the possibility that there are features of these two glycoproteins or plasmids which reduce the efficiency of incorporation of these glycoproteins into PT particles.

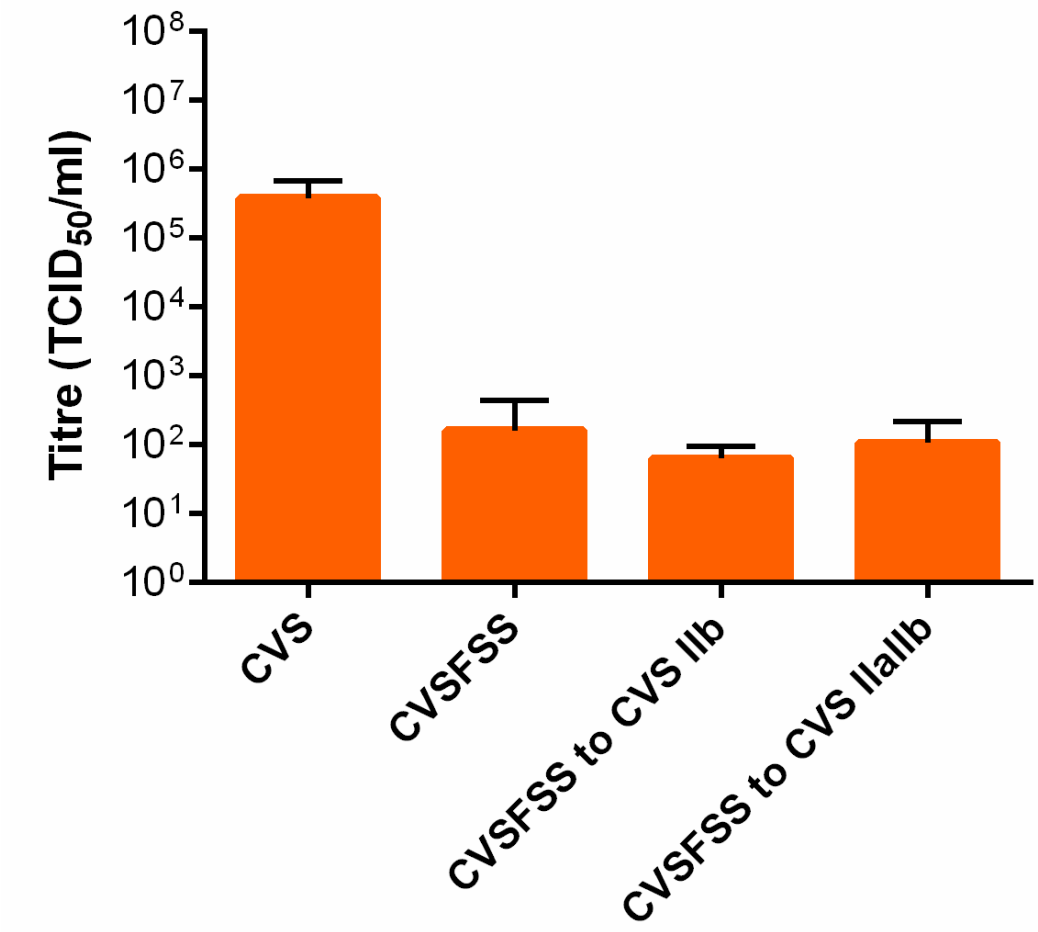


Figure 3.5: Titres of pseudotypes containing mutations to try and increase the titre of CVSFSS The CVSFSS PT construct was mutated back to wildtype sequence across the site II antigenic site to attempt to increase titre. Error bars represent standard deviation (SD) about the mean of three replicate in-assay titrations.

3.7 Characterisation of PT particles

Once the panel of PT viruses had been generated and titres determined, further characterisation of these particles was conducted. In order to determine the size of the PT particles CVS PT was visualised using a NanoSight instrument (Malvern). This instrument is able to visualise particles based on the light scattered when illuminated by laser light. The scattered light is tracked from frame to frame by specialised software which enables calculation of the particle size based on the assumption of Brownian motion in a liquid. Both CVS PT and a β-propiolactone inactivated preparation of RABV were measured alongside each other on the NanoSight. The size distribution of both preparations is shown in Figure 3.6.

The average size of particles in the inactivated RABV virus suspension was 168.2 nm with a standard deviation (SD) of 58.3 nm whereas the spread of particle size in the PT preparation was much greater. The average CVS PT particle size was 266.6 nm with a SD of 125.2 nm. The size of a rabies virion is known to be approximately 60 nm x 180 nm (Rupprecht, 1996) and this has been confirmed by the RABV average size of 168.2 nm. The size of PTs appears to be generally larger than virus particles and much more variable.

Having estimated particle size, the expression of G on PT membranes was assessed. Initially, flow cytometry was utilised to detect fluorescent antibody tagged PT particles alongside the HEK 293T cells which had generated the PTs. Again, as the type species for RABV the CVS PT present in harvests from the supernatant of transfected cells and HEK 293Ts transfected to produce CVS PT were analysed. Both the CVS PT and CVS HEK 293T cells were exposed to a non-fluorescently tagged rabies G specific mAb

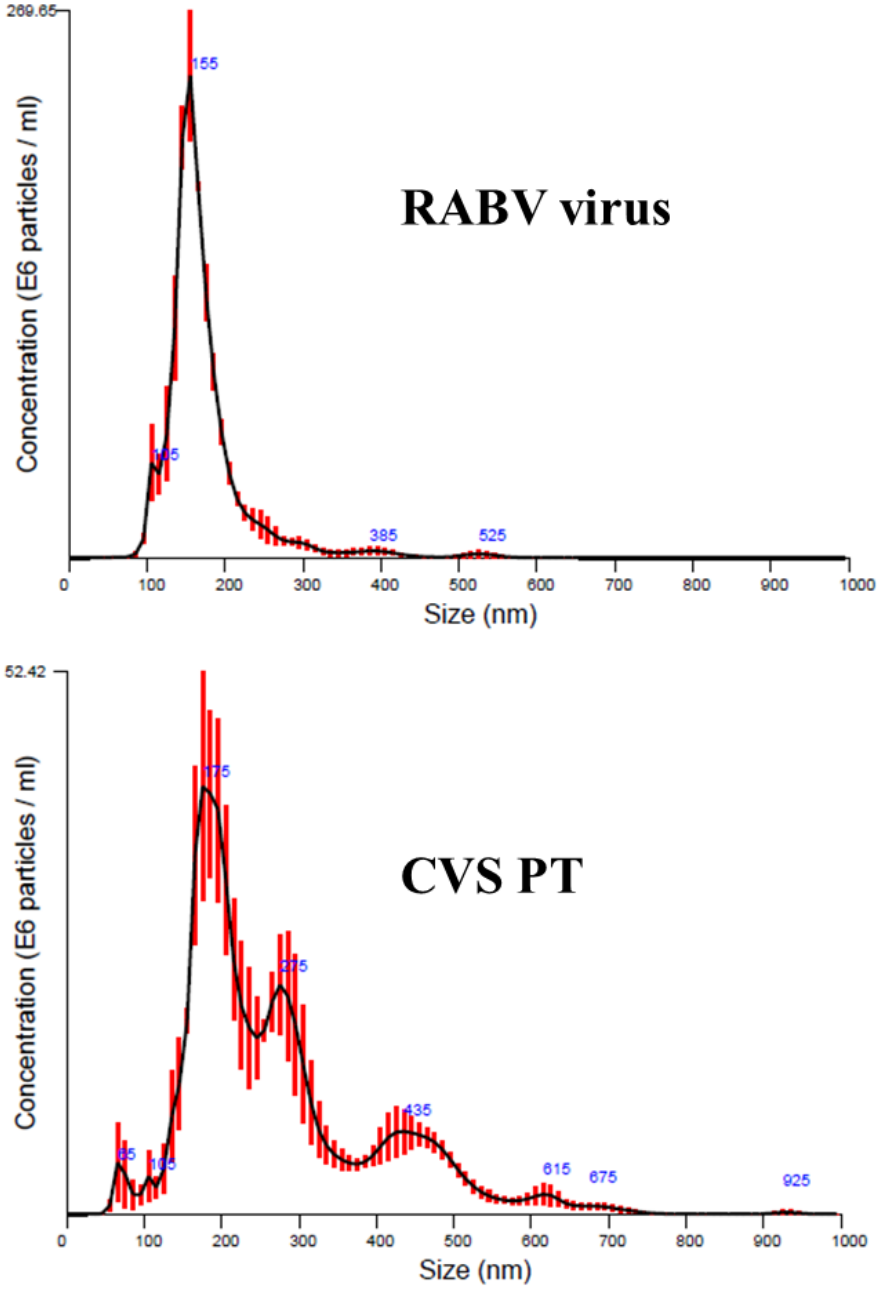


Figure 3.6: NanoSight measurement of CVS PT and RABV virus particles.

Suspensions of inactivated RABV and CVS PT were detected. The range of particle sizes is shown.

alongside tissue culture supernatant (SN) from untransfected cells and untransfected HEK 293T cells as negative controls. After incubation with the primary antibody this was washed off and replaced with an Alexa Fluor 568 tagged secondary antibody to enable indirect staining of the PTs and cells in the red light spectrum. Density plots of the events measured can be seen in Figure 3.7.

It was clear that more events were measured with the cell containing samples than with the PT containing samples. In addition the forward scatter of cells versus PTs shows that PT particles are much smaller than cells, as expected. The staining of the cells was much more successful than staining of the PT particles. It was clear that no staining of PT particles occurred as the gate over the majority of the population shows the same number of events in both the CVS PT and cell SN. In contrast the cell staining showed a clear difference between the two different cell samples. The staining of the un-transfected cells showed only 0.05% of the gated population positive for fluorescence whereas the CVS HEK 293T cell population had 46.39% positive for fluorescence. This suggested that the staining of the cells was successful and that approximately half of the samples cells were expressing G on their surface which indicated a transfection efficiency of approximately 50%.

Once the PT particles and the cells expressing them had been detected by flow cytometry, the presence of G on the cell surface was also confirmed by immunofluorescence using the same primary and secondary antibodies as used in the flow cytometry experiment. A selection of images from the panel of PTs are displayed in Figure 3.8. Figure 3.8 A to C show the control staining conducted. It was apparent

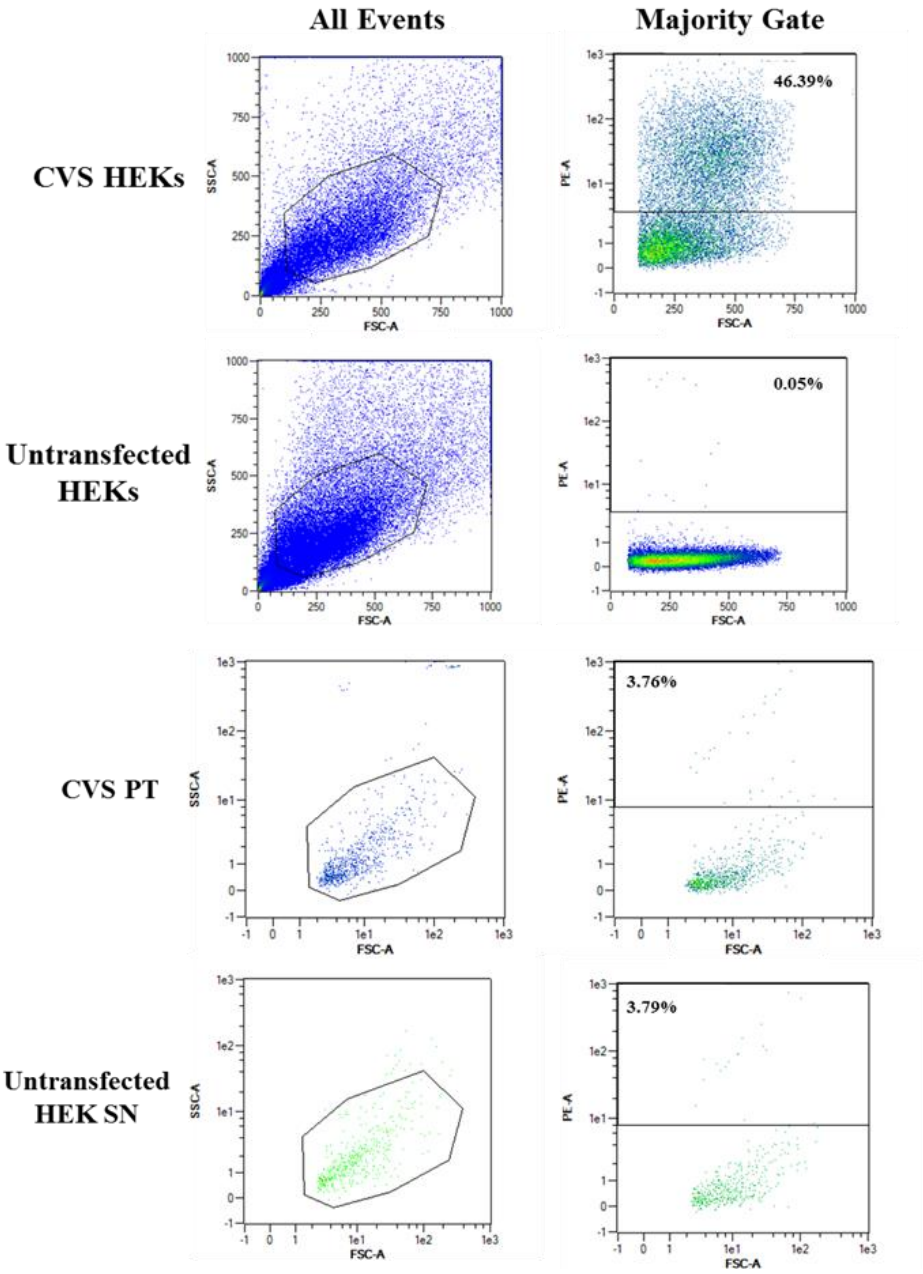


Figure 3.7: Detection of lyssavirus glycoprotein on cells and PTs by flow cytometry. The majority of the cell and PT populations were gated from the total events detected and representative dot plots of indirect anti-glycoprotein staining of cells transfected to produce CVS PT, CVS PT, un-transfected cells and un-transfected cell supernatant are shown. The primary antibody was a murine anti-RABV G mAb (MyBioSource) at a 1 in 50 dilution and the secondary antibody as goat anti mouse Alex Fluor 568 conjugated antibody at a 1 in 200 dilution (Life Technologies).

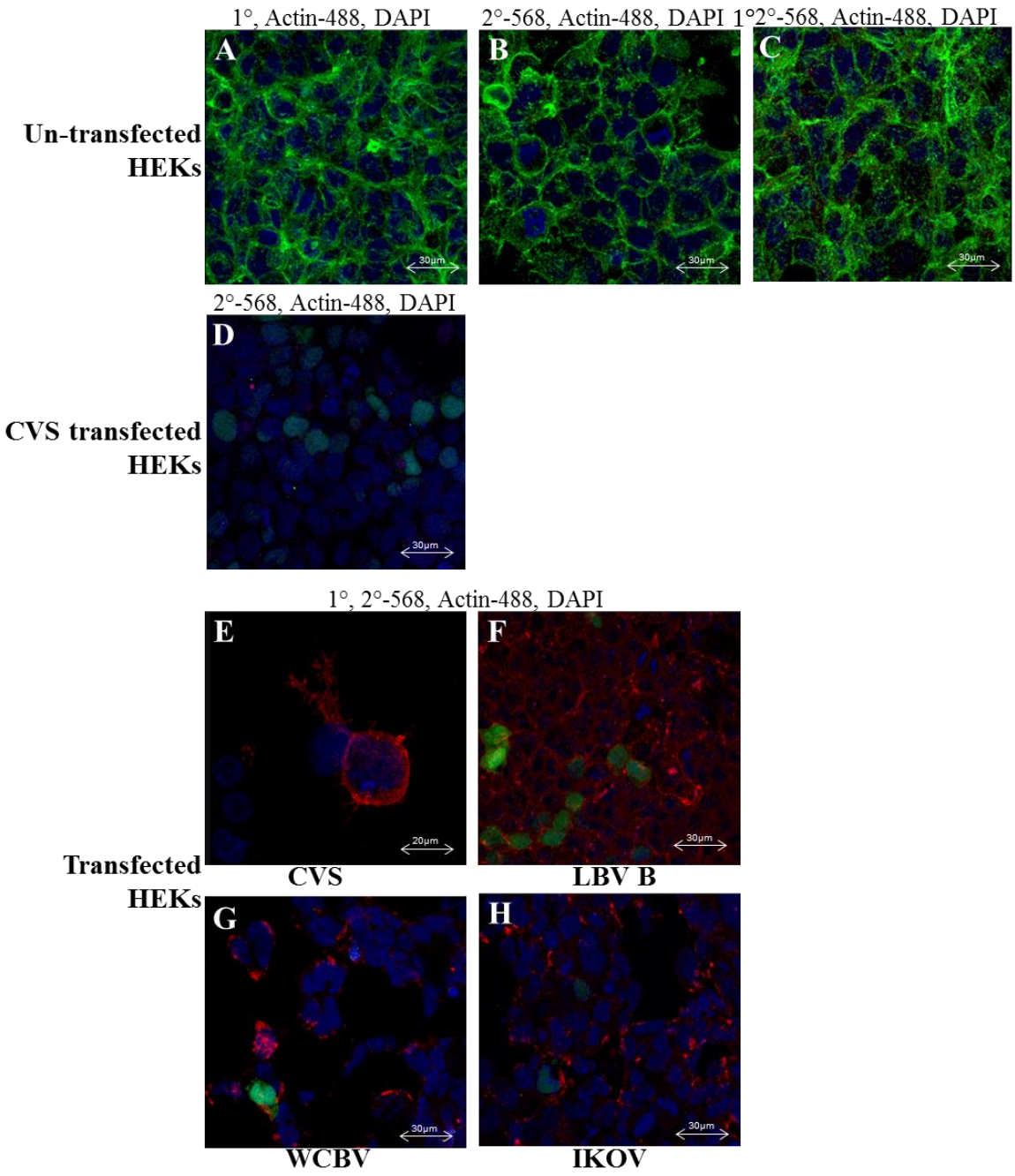


Figure 3.8: Immunofluorescence detection of lyssavirus glycoprotein post transfection. (A-C) Untransfected HEK 293T cells. (A) Stained with primary, actin-phalloidin and DAPI antibodies. (B) Stained with secondary, actin-phalloidin and DAPI antibodies. (C) Stained with primary, secondary, actin-phalloidin and DAPI antibodies. (D) HEK 293T cells transfected to produce CVS PT, stained with secondary, actin-phalloidin and DAPI antibodies. (E-F) Transfected HEK 293T cells stained with primary, secondary, actin-phalloidin and DAPI antibodies. (E) Cells transfected to produce CVS PT. (F) Cells transfected to produce LBV B PT. (G) Cells transfected to produce WCBV PT. (H) Cells transfected to produce IKOV PT. All images presented are z-stacks. Primary mAb used was anti- RABV G antibody at a 1/50 dilution (MyBioSource), the secondary was Alexa-fluor goat anti mouse mAb (Red) at a 1/200 dilution (Invitrogen) and DAPI Vectashield (Vectorlabs) was used to stain the cell nuclei. The actin-phalloidin mAb (green) was used at a 1/50 dilution (Invitrogen).

that there was no non-specific fluorescence with either the untagged primary antibody alone (Figure 3.8 (A)) or the 568-tagged secondary antibody alone (Figure 3.8 (B)), nor in combination (Figure 3.8 (C)). Figure 3.8 (D) shows unusual actin-phalloidin staining in cells transfected to produce the CVS PT. This unusual actin staining was apparent in every transfection image, however the unusual staining was not present in every cell. The reason for this effect on actin staining remains unclear. Despite the unusual actin-phalloidin staining the DAPI and glycoprotein staining were more successful. Figure 3.8 images E to H show a panel of cells transfected to produce a variety of different lyssavirus PTs. The pattern of glycoprotein staining appears to be specific to the surface of the HEK 293T cells with some cytoplasmic staining. The cell surface staining was particularly evident in the CVS image (Figure 3.8 (E)) however the same pattern was evident for the more divergent PTs (Figure 3.8 (F to H)). It was interesting that the staining appeared to reduce in intensity with increasing genetic divergence away from CVS (Figure 3.8 (E)) as LBV B (Figure 3.8 (F)) is stained more strongly than WCBV (Figure 3.8 (G)) and IKOV (Figure 3.8 (H)).

Finally, detection of glycoprotein in transfected cells was assessed by Western blot. The same mAb utilised for flow cytometry and immunofluorescence was used to detect G in cell lysates. HEK 293T cells transfected to produce the panel of PTs were lysed and the concentration of input cell lysate and dilution factor of the mAb were optimised. A dilution of cell lysate 1 in 10 and the mAb 1 in 1000 was found to produce the clearest blots (data not shown). Once the mAb had been optimised for use in Western blots the panel of cell lysates were run on denaturing gels prior to probing with the mAb and subsequent development of the blot. The result of the Western blots can be seen in Figure 3.9.

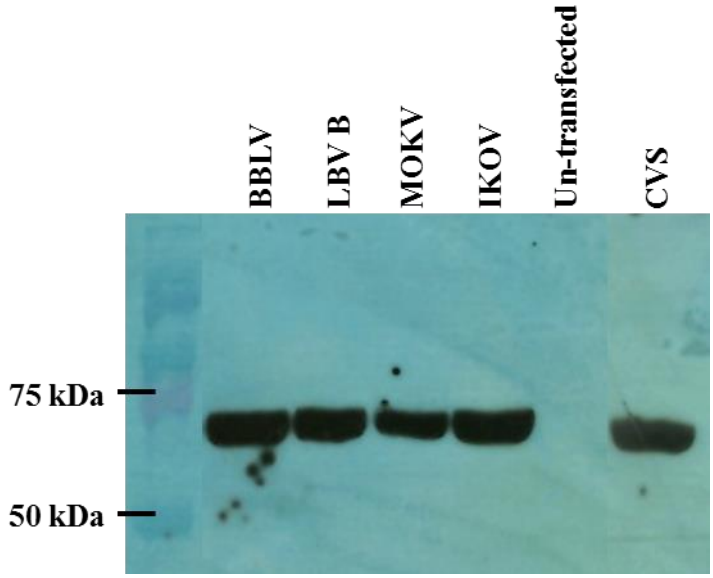


Figure 3.9: Western blot of a panel of cell lysates from cells transfected to produce lyssavirus PTs. The primary antibody was the anti- RABV G antibody (MyBioSource) at a 1/1000 dilution. The secondary was polyclonal rabbit anti mouse immunoglobulins/HRP (Dako) at a 1/1000 dilution. The glycoprotein of 65 kDa was visible.

The lyssavirus glycoprotein is 65 kDa (Maillard & Gaudin, 2002) and this can be seen for a number of PTs in Figure 3.9.

These characterisation experiments have shown that PT particles appear to be larger than lyssavirus virions and that the glycoprotein of lyssavirus PTs can be detected on the surface of transfected cells by both flow cytometry and immunofluorescence. PT particles themselves can be detected by flow cytometry but the mAb used in this investigation was unable to detect glycoprotein on the surface of PT particles. The same mAb was however able to detect wildtype lyssavirus glycoproteins on transfected cells by IF and via Western blot.

3.8 Discussion

A large panel of both wt and mutant lyssavirus PTs has been generated by the use of overlap extension mutagenesis, standard ligation cloning techniques and commercial gene synthesis.

It is apparent that the titres vary between PTs, regardless of the presence of antigenic site mutations. The wildtype PTs do, however tend to achieve higher titres and this may be due to the lack of mutations within the G. Several G expressing constructs were unable to generate measurable PT titres, including RV61 and RV193 (data not shown) along with LBV D and CVSFSS that both gave very low titres. The reason for this remains undefined although several factors may be involved including the potential for variation between plasmid preparations with respect to salt concentrations which may affect the transfection efficiency by modulating the membrane rigidity of the liposomal formations (Bouvrais, 2012). Multiple plasmid preparations were utilised in repeat attempts to

generate LBV D and CVSFSS PTs, both of which produced very low titres however these two PTs could not be generated to useable titres.

As described in Section 3.6, attempts to improve the titre of the CVSFSS PT by reverting the apparently deleterious site II mutations back to wildtype sequence were also unsuccessful (Figure 3.5). The crystal structure of the lyssavirus glycoprotein remains undefined which makes interpretation of some of the antigenic site mutant titration failures challenging. The absence of a crystal structure for the RABV G precludes structural assessment of the impact of antigenic site swaps so it remains unclear whether the interactions between sites IIa and IIb play a role in the correct folding and proper function of the mature protein. It has been suggested in a proposed two-dimensional structure of the rabies glycoprotein that sites IIa and IIb are located in close proximity within the primary structure of the protein (Walker & Kongsuwan, 1999) so perhaps by altering residues in these areas for the CVS to LBV IIa and IIb mutants along with the CVSFSS to CVS IIb mutant this affected the interactions between sites IIa and IIb which resulted in incorrect folding. There were also variations between titres of many of the CVS and LBV site swap mutants which may also correspond to changes in the amino acid sequences and thus protein folding.

Each amino acid has specific physiochemical properties which confer either favourable, non-favourable or neutral substitution values if one amino acid is switched for another in the same position (Betts & Russell, 2003). The physiochemical substitution scores for each amino acid switch between the antigenic sites of CVS and LBV are shown in Table 3.2. All of the substitution scores were negative, i.e. none of the antigenic site swaps were favourable, however some site swaps were less favourable than others. Within

Table 3.2: The substitution scores based on physiochemical properties of amino acids of the antigenic site swaps between CVS and LBV in an extracellular context. The score is identical for the LBV to CVS reciprocal swap. (Betts & Russell, 2003).

	Site IIb									Site IIa			Site I						Site IV		Site III							
CVS	G	C	T	N	L	S	E	F	S	K	K	A	K	L	C	G	V	L	F	H	K	S	V	R	T	W	N	E	I
LBV	G	C	G	T	S	S	V	F	S	K	K	S	T	L	C	H	K	P	N	R	L	K	V	D	N	W	S	E	I
Score (-ve)	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	1	0	0	2	0	0	0	0	0

antigenic site IIb there was only one unfavourable change; the leucine (L) to serine (S) switch was marginally unfavourable as L is usually found within the interior of a protein, unlike S. However, S is weakly polar and so may be involved in defining a proteins' structure. The substitution in antigenic site IIa was neutral in an extracellular context, as with the substitutions in antigenic site I. Antigenic site IV is the smallest antigenic site however it has a relatively unfavourable switch of phenylalanine (F) to asparagine (N). F is hydrophobic so generally found in the hydrophobic core of proteins whereas N is polar so a position on the surface of proteins is more favourable. This may explain why the swap was unfavourable as the two amino acids have opposing physiochemical properties. Antigenic site III contained the most unfavourable changes. The positive polar lysine (K) was switched with the hydrophobic L whilst the other positive polar residue, arginine (R) was switched for a negative polar residue, aspartic acid (D) thus potentially explaining the unfavourable change as the two residues have opposing charges.

These substitution values for each of the antigenic sites may play a role in the folding of the glycoprotein and therefore the functionality which may have directly affected formation of the PT particles and their viability. Therefore the relationship between the substitution values of each antigenic site and the titre of the corresponding PT was investigated. The relationship of both the CVS and LBV site swap PT panels with the site specific substitution values can be seen in Figure 3.10.

There is no clear correlation between titre and substitution value and both the P and R2 values for the CVS and LBV data both confirm there is no significant correlation between titre and substitution value. This suggests that the physiochemical consequences

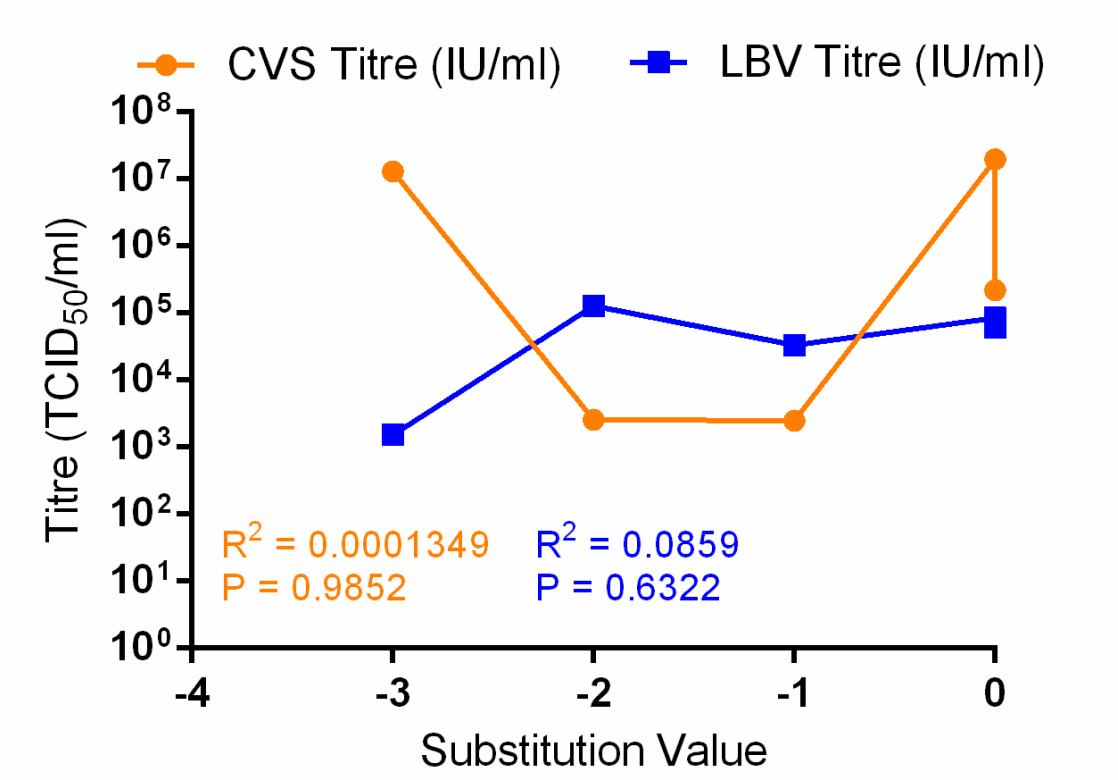


Figure 3.10: The relationship between amino acid substitution value and titre of the corresponding antigenic site mutants of both CVS and LBV. The substitution values were determined according to (Betts & Russell, 2003) and the correlation was determined between each PT titre and the corresponding substitution value using GraphPad Prism 6.

of the alterations in antigenic site sequences in the mutant PTs did not have an effect on the titre. This means that there may be other features of the mutant glycoproteins which affect the titre of the resulting PT, for example the residues surrounding the antigenic sites may play a role (see Section 6.5). This can be seen in the titres of CVSFSS, CVS to LBV IIaIIb and CVSFSS to CVS IIaIIb as all only achieved low titres, despite both CVS wt sites IIa and IIb being present. Until the crystal structure of the glycoprotein is deduced however, these hypotheses cannot be confirmed.

In vitro characterisation of these PT particles using a range of different techniques was conducted and visualisation of PT particles was attempted using both NanoSight technology and flow cytometry. The NanoSight data suggested that the sizes of PT particles are generally larger than virions and much more variable.

The presence of glycoprotein on the surface of transfected HEK 293T cells was detected by flow cytometry which indicated a transfection efficiency of approximately 50%. Though PTs could be detected by flow cytometry, the fluorescence staining of the PTs was unsuccessful. This may be because the binding epitope of the available mAb requires mature conformation for binding. The conformation of this mAb epitope on the surface of transfected cells may be different to that on the PT plasma membrane, thus resulting in a lack of staining, potentially due to a lack of G expression on the PT surface or presentation of G monomers as opposed to trimers.

The protein preparations were run on denaturing gels for Western blot analysis which may have affected the epitope of the mAb so it would be interesting to run these lysates on a non-denaturing gel to determine whether this affects G staining. In addition an

alternative mAb may be used in to stain PTs for flow cytometry as this could be a very valuable tool for the assessment of PT particles. Alternatively a lower dilution of the mAb could be attempted as although a 1 in 50 dilution was recommended by the manufacturer, due to the small number of PT particles measured an increase in mAb availability may improve staining.

Regardless, a panel of wildtype and mutated PT particles had been generated and could now be assessed using neutralisation assays to evaluate the ability of different sera to neutralise the different PT particles (Chapter 4).

Chapter 4: Investigation into the neutralisation profiles of a panel of lyssavirus pseudotypes

4.1 Introduction

A panel of pseudotype particles (PTs) were generated representing glycoproteins from each of the lyssavirus species alongside PTs that contain mutations to immunogenic antigenic sites within the glycoprotein (Chapter 3). The fact that the current rabies vaccines are all based on classical rabies virus strains and are unable to generate a neutralising response that protects against all lyssaviruses led to an investigation into the ability of hyperimmune sera from immune donors to neutralise the various lyssavirus PTs. Here we describe attempts to understand the neutralisation of different PTs by hyperimmune sera derived from different species.

4.2 Optimisation of the PNA

Once the panel of PTs had been generated they were available for assay. Initially the PT neutralisation assay required optimisation for use with firefly luciferase; the reporter used in this investigation. Two luciferase assay systems were compared; the Luciferase Assay System (Promega) and Bright-Glo (Promega). The Luciferase Assay System required a substantially more complex setup than Bright-Glo and the read time per plate was up to 25 minutes as opposed to just two minutes with Bright-Glo, thus the Bright-Glo system was adopted for the course of this investigation.

In addition to luciferase assay system optimisation, input of PT virus was also optimised. The standard practice in a PT assay is to use a quantified concentration of PT, either assayed by relative light unit (RLU) or by titre. This requires the dilution of PT such that

the input PT virus is approximately equal. A negative cut-off value was assigned as the average RLU of at least 4 wells of cells plus media multiplied by 2.5 (Wright *et al.*, 2008). Following optimisation of the assay consistent results on back titration were achieved by diluting PT to 400 $TCID_{50}$/ml and then addition of 50µl of this per well. This resulted in a fairly consistent back titration of 200 $TCID_{50}$/ml. A back titration of each PT dilution was performed alongside each assay as a control for PT input. If any back titres differed substantially from 200 $TCID_{50,}$ the assay was repeated. A further control was the addition of PT to cells in the absence of sera to give a value for RLU from untreated PT. This control generated a value from which a percentage neutralisation was calculated and compared to those treated with the different test sera. A negative dog serum control was also tested to confirm that the reduction in luminescence seen with test sera was as a result of specifically neutralising antibodies present in the test sera which are absent from naïve dog serum.

4.3 Neutralisation within phylogroup I

It has been suggested that there is a variable degree of cross neutralising antibody response generated following vaccination with standard rabies vaccines and that the titre required to neutralise phylogroup I lyssaviruses may vary (Brookes *et al.*, 2006). Since this initial publication, novel lyssaviruses that are genetically classified within phylogroup I had been discovered and so it was of interest to investigate the ability of sera from vaccinated animals and humans to neutralise the full range of these viruses. Two types of hyperimmune sera were assessed: WHO serum, an international standard sera prepared from blood sampled from vaccinated humans that is used as control sera for WHO gold standard diagnostic assays; and VLA serum, a diagnostic control serum obtained from a pool of vaccinated dogs used in serological diagnostic tests performed at

the Animal and Plant Health Agency, UK, as a control sera for neutralisation tests. Both sera were diluted to 0.5 IU/ml which is the WHO and OIE internationally accepted cut-off titre for protection according to dilutions assessed using live virus in the UKAS accredited Fluorescent Antibody Virus Neutralisation test (FAVN). For each PT, the preparation of PT was diluted out to 200$TCID_{50}$/ml and incubated with 50µl of either WHO or VLA serum and assayed according to Section 2.3.3. The results of this assay can be seen in Figure 4.1.

It is clear that all of the PTs were very strongly neutralised by both the WHO human and the VLA dog sera at 0.5 IU/ml so the PT panel were run against 0.1 IU/ml of both sera in order to determine whether the cross neutralisation was conserved following a 5-fold dilution of control hyperimmune sera (Figure 4.2 A).

All of the phylogroup I PTs were strongly neutralised by both WHO and VLA sera at 0.1 IU/ml as well as 0.5 IU/ml suggesting that there is a considerable degree of cross neutralisation within phylogroup I, both to a protective titre of anti-RABV serum but also to an apparently sub-protective but clearly neutralising dilution of serum.

The next stage was to investigate whether there was any cross neutralising capacity by phylogroup II and III specific sera against the phylogroup I PTs. The same panel of PTs used in Figures 4.1 and 4.2 were run against a panel of rabbit sera directed against LBV B, a phylogroup II lyssavirus, WCBV, a phylogroup III lyssavirus and IKOV, a novel highly divergent lyssavirus (see Section 5.5). The results of the assay can be seen in Figure 4.3.

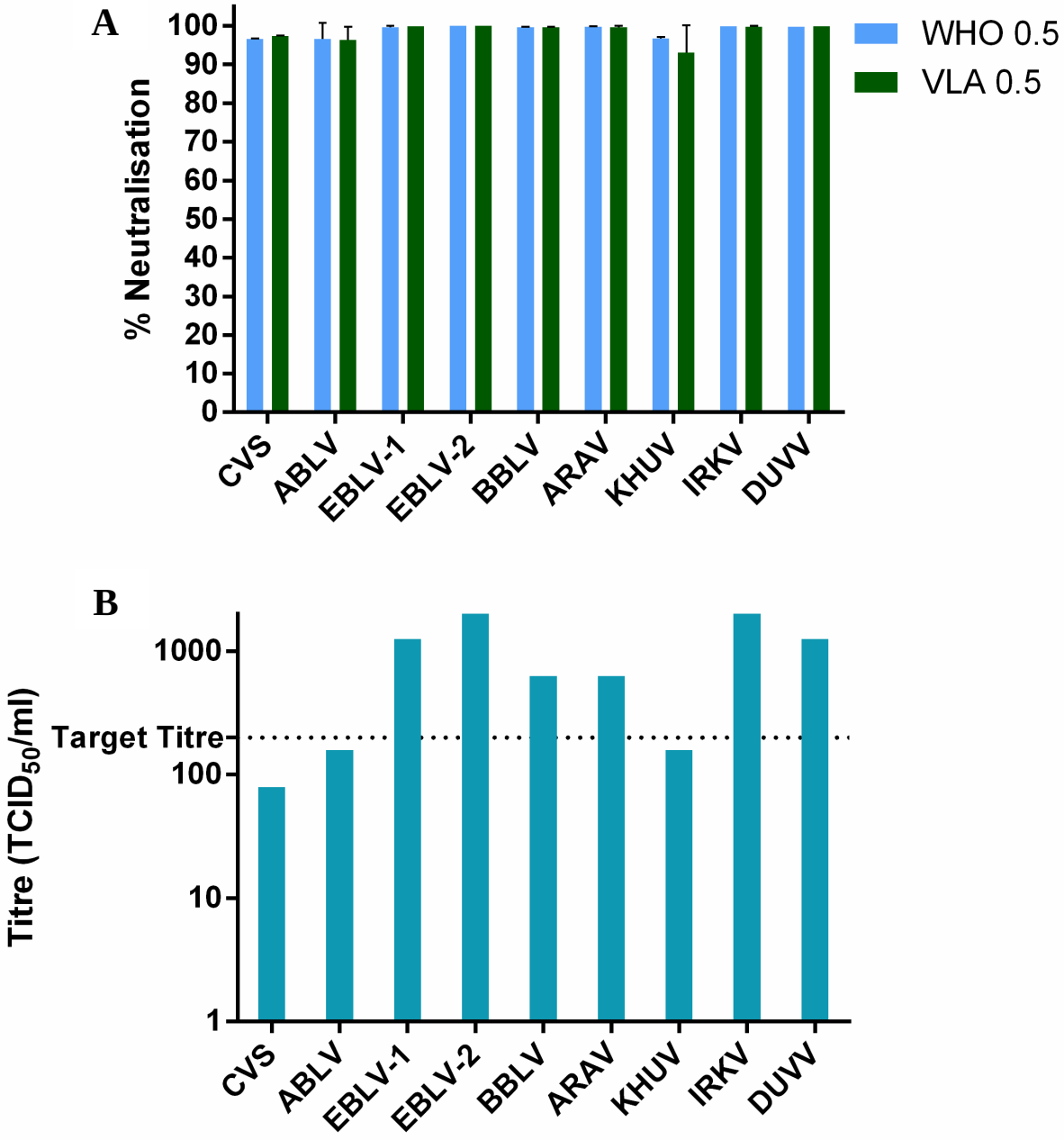


Figure 4.1: Neutralisation profile of phylogroup I PTs against 0.5 IU/ml RABV specific sera. (A) PT neutralisation assay. WHO serum from vaccinated human and VLA serum from vaccinated dogs were diluted to 0.5 IU/ml. (B) Back titrations of each PT used in the assay. The dotted line indicates the target titre following PT dilution. Error bars represent standard deviation (SD) about the mean of three replicate in-assay neutralisation tests.

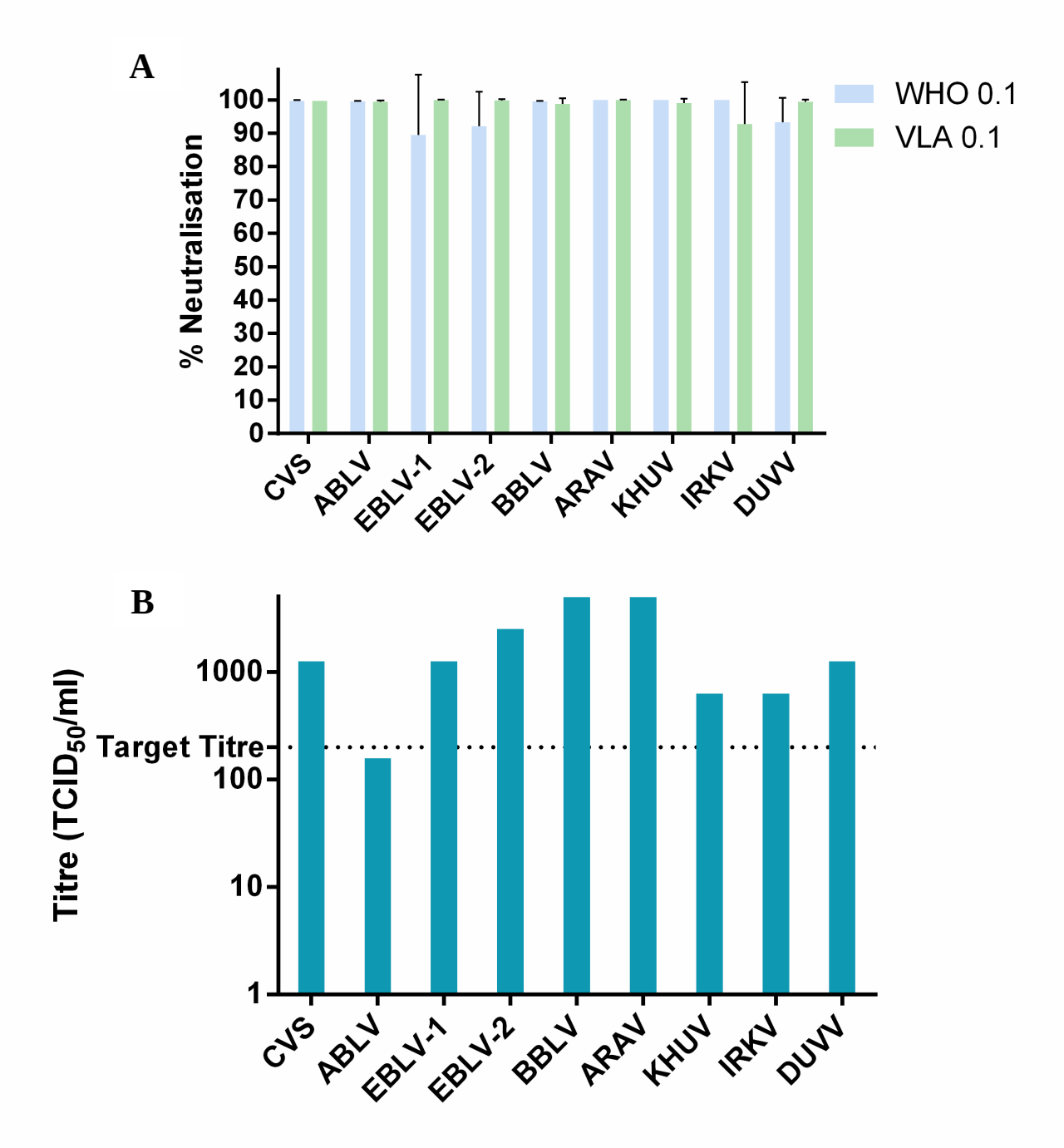


Figure 4.2: Neutralisation profile of phylogroup I PTs against 0.1 IU/ml RABV specific sera. (A) PT neutralisation assay. WHO serum from vaccinated human and VLA serum from vaccinated dogs were diluted to 0.1 IU/ml. (B) Back titrations of each PT used in the assay. The dotted line indicates the target titre following PT dilution. Error bars represent standard deviation (SD) about the mean of three replicate in-assay neutralisation tests.

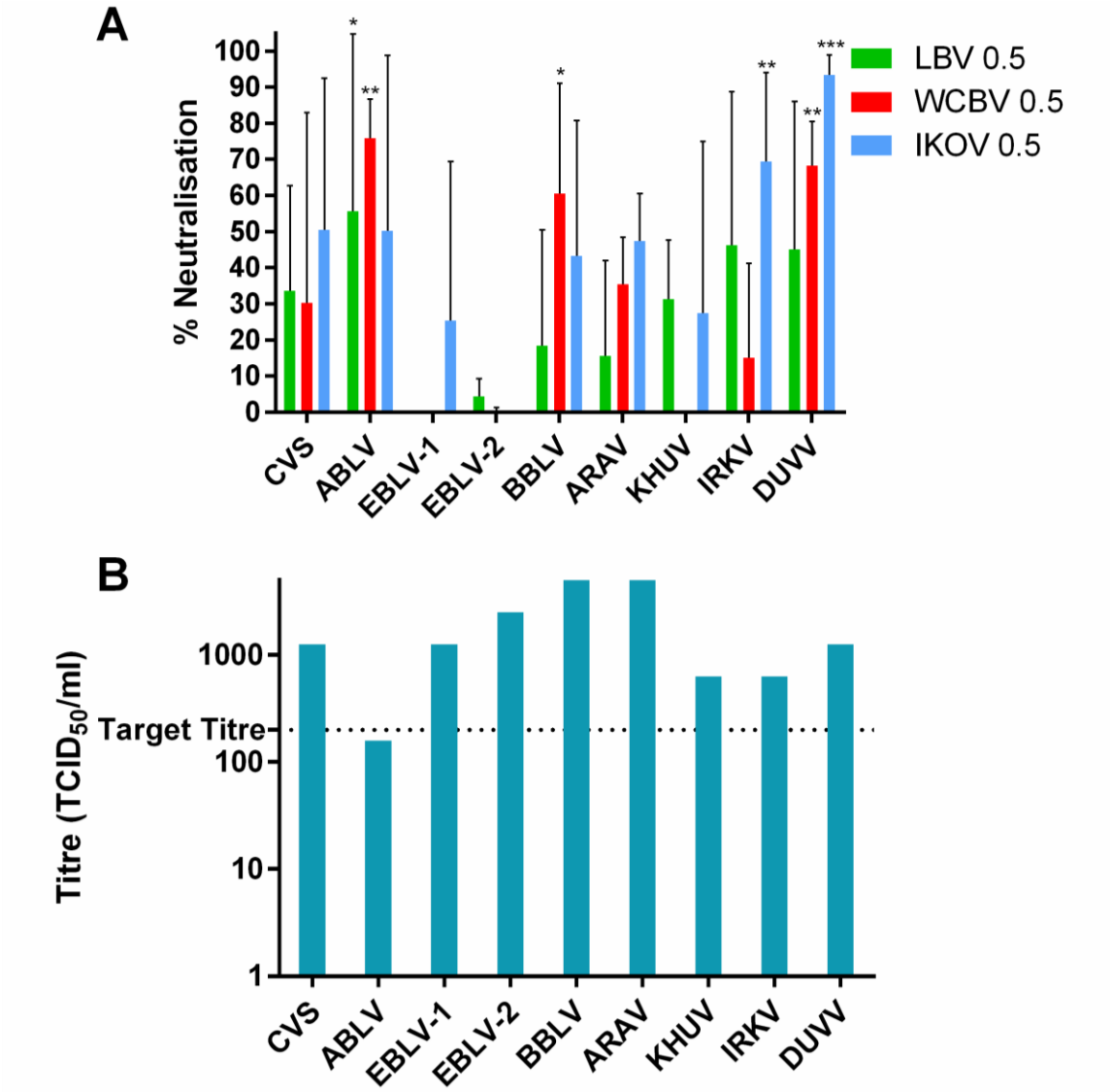


Figure 4.3: Neutralisation profile of phylogroup I PTs against phylogroup II and III specific sera. (A) PT neutralisation assay. LBV B serum represents phylogroup II, WCBV serum represents phylogroup III and IKOV serum represents a highly divergent lyssavirus. All sera were diluted to 0.5 IU/ml. Error bars represent standard deviation (SD) about the mean of three replicate in-assay neutralisation tests. A 2-way ANOVA was used to determine any significant differences between neutralisation of each PT by the test sera compared to the lack of neutralisation by negative dog serum (0% neutralisation by negative dog serum data not shown). Significant differences are indicated by asterisks; *$p \leq 0.05$, **$p \leq 0.005$, ***$p \leq 0.001$. (B) Back titrations of each PT used in the assay. The dotted line indicates the target titre following PT dilution.

The majority of the PTs were not significantly neutralised by any of the three divergent sera however ABLV appeared to be significantly neutralised by the anti-WCBV by 76%. Likewise BBLV was significantly neutralised by the WCBV by 60.5% and DUVV by 68%. DUVV was also significantly neutralised by the anti-IKOV by 93% whilst IRKV was also significantly neutralised by the IKOV serum by 69%. These data suggest that there may be some level of cross neutralisation with sera generated against the most divergent lyssaviruses (WCBV and IKOV) being able to neutralise some phylogroup I PTs; however the neutralising capacity of divergent lyssavirus specific sera against phylogroup I PTs appears to be specific for select PTs as the neutralisation profiles of all three sera were not equal across the PT panel.

4.4 Investigation of neutralisation within phylogroup II

Having observed a high degree of cross phylogroup I neutralisation (Figures 4.1 and 4.2) and a degree of neutralisation of some phylogroup I lyssaviruses with sera specific to divergent phylogroup II and III lyssaviruses (Figure 4.3) the next stage was to assess the ability of phylogroup II specific sera to neutralise PT containing representatives from each of phylogroups II and III. This was investigated using a panel of phylogroup II PTs and LBV B specific serum (Figure 4.4). Representatives of three of the four lineages of LBV were included due to the antigenic divergence between these isolates along with the other characterised African lyssaviruses. The result of this assay is detailed in Figure 4.4. Despite numerous efforts to rescue the titre of the LBV D PT, it did not reach the threshold for utilisation in the PNA (Section 2.3.3) and as such could not be included. The panel of phylogroup II and III PTs was run against an LBV B specific rabbit derived polyclonal serum in order to determine the degree of cross neutralisation.

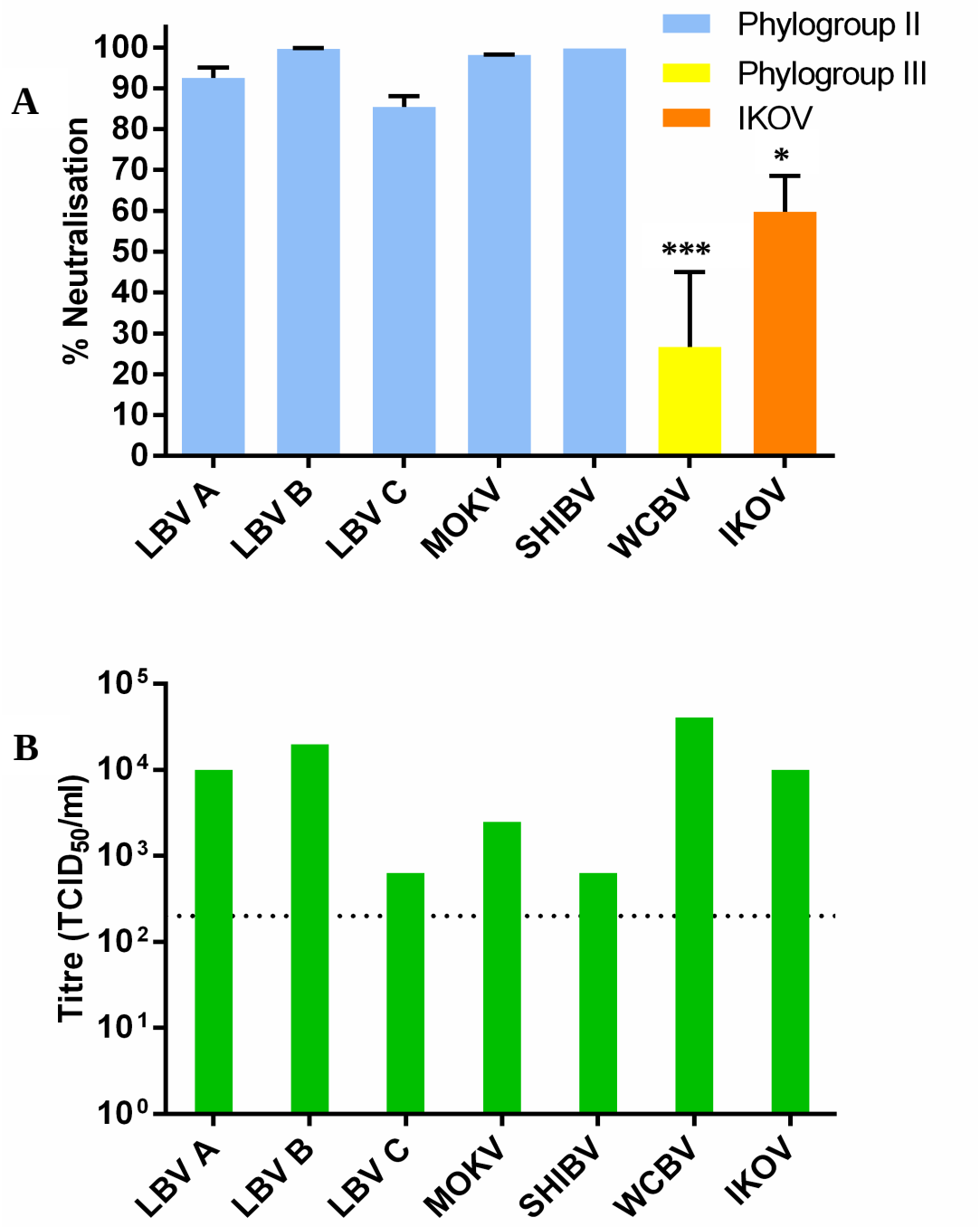


Figure 4.4: Cross neutralisation within a phylogroup II lyssavirus PT panel. (A) PT neutralisation assay. PTs representing all members of phylogroup II plus phylogroup III WCBV and the highly divergent IKOV were run against an LBV B specific rabbit derived polyclonal serum diluted to 0.5 IU/ml. A 2-way ANOVA was used to determine any significant differences between neutralisation of each PT by LBV B serum and LBV B PT by LBV B serum. Significant differences are indicated by asterisks; *$p \leq 0.05$, ***$p \leq 0.001$. (B) Back titrations of each PT used in the assay. The dotted line indicates the target titre following PT dilution. Error bars represent standard deviation (SD) about the mean of three replicate in-assay neutralisation tests.

In Figure 4.4 the phylogroup II PTs can be seen in pale blue. There was a strong degree of neutralisation of all of these PTs by the LBV B serum which suggested a significant degree of cross neutralisation within this group of antigenically and genetically similar viruses. WCBV is more divergent than the phylogroup II viruses, having only 54% amino acid identity with LBV B, so a low degree of cross neutralisation was expected and this was confirmed with WCBV being only 27% neutralised by the LBV B specific sera. Likewise the IKOV glycoprotein is more divergent still than WCBV, with just a 48% amino acid identity with LBV B, so a lower degree of neutralisation was expected, however, IKOV was 60% neutralised by the LBV B serum. This degree of neutralisation was unexpected although it is significantly lower than the degree of neutralisation between PTs within phylogroup II. This experiment confirms that, as observed between the viruses in phylogroup I (Brookes *et al.*, 2006; Brookes *et al.*, 2005) there was a high degree of cross neutralisation between viruses in phylogroup II. This supports the concept of including only one phylogroup I and one phylogroup II lyssavirus glycoprotein in any cross protective antigen formulation as these should be able to provide sufficient cross neutralisation between all viruses in phylogroups I and II.

4.5 Investigation of cross neutralisation between phylogroups

Following establishment of the level of cross neutralisation within phylogroups it was of interest to determine the level of cross neutralisation between phylogroups. It has long been acknowledged that the current rabies vaccines are unable to produce an antibody response that can neutralise viruses from other phylogroups efficiently. Most recently, with the discovery of Ikoma virus a complete lack of cross reactivity of high titre human and pet hyperimmune sera for IKOV has been demonstrated (Horton *et al.*, 2014). Alongside this, mice vaccinated with rabies vaccines that seroconverted were also

completely unprotected following challenge with IKOV (Horton *et al.*, 2014). In order to investigate this further, representatives of each phylogroup were selected on the basis that the neutralisation profiles within phylogroups appear to be similar. This panel of PTs was then exposed to a range of sera at different concentrations in order to investigate any inherent cross neutralisation.

Initial data in Figures 4.1 and 4.3 suggested that 0.5 IU/ml of sera was sufficient to neutralise all members of phylogroup I however there was limited cross neutralisation with 0.5 IU/ml of phylogroup II and III sera against phylogroup I PTs. For this reason a series of increasing concentrations of RABV specific sera were run against the representatives of all phylogroups in order to determine whether this induced neutralisation of divergent lyssavirus PTs. The results of this PNA can be seen in Figure 4.5. The sera tested were the same as those utilised previously (WHO and VLA) and due to their high concentration these sera could be diluted out to a range of IU/ml to assess the ability of increasing concentrations of sera to neutralise each PT.

The percentage neutralisation of each PT was compared to that of CVS for each serum dilution using a two way ANOVA test and a level of significance (alpha value) of 0.05. Those values which significantly differ from CVS are indicated by asterisks on Figure 4.5. At each concentration of both WHO and dog serum LBV B neutralisation was not significantly different to CVS. The WHO serum was able to neutralise WCBV and IKOV to an extent at both 5 IU and 10 IU however at 5 IU the neutralisation of WCBV was not significantly different to CVS suggesting substantial neutralisation, though this pattern was not continued at 10 IU. The dog serum was not able to significantly neutralise either WCBV or IKOV at any concentration however at 10 IU a small degree

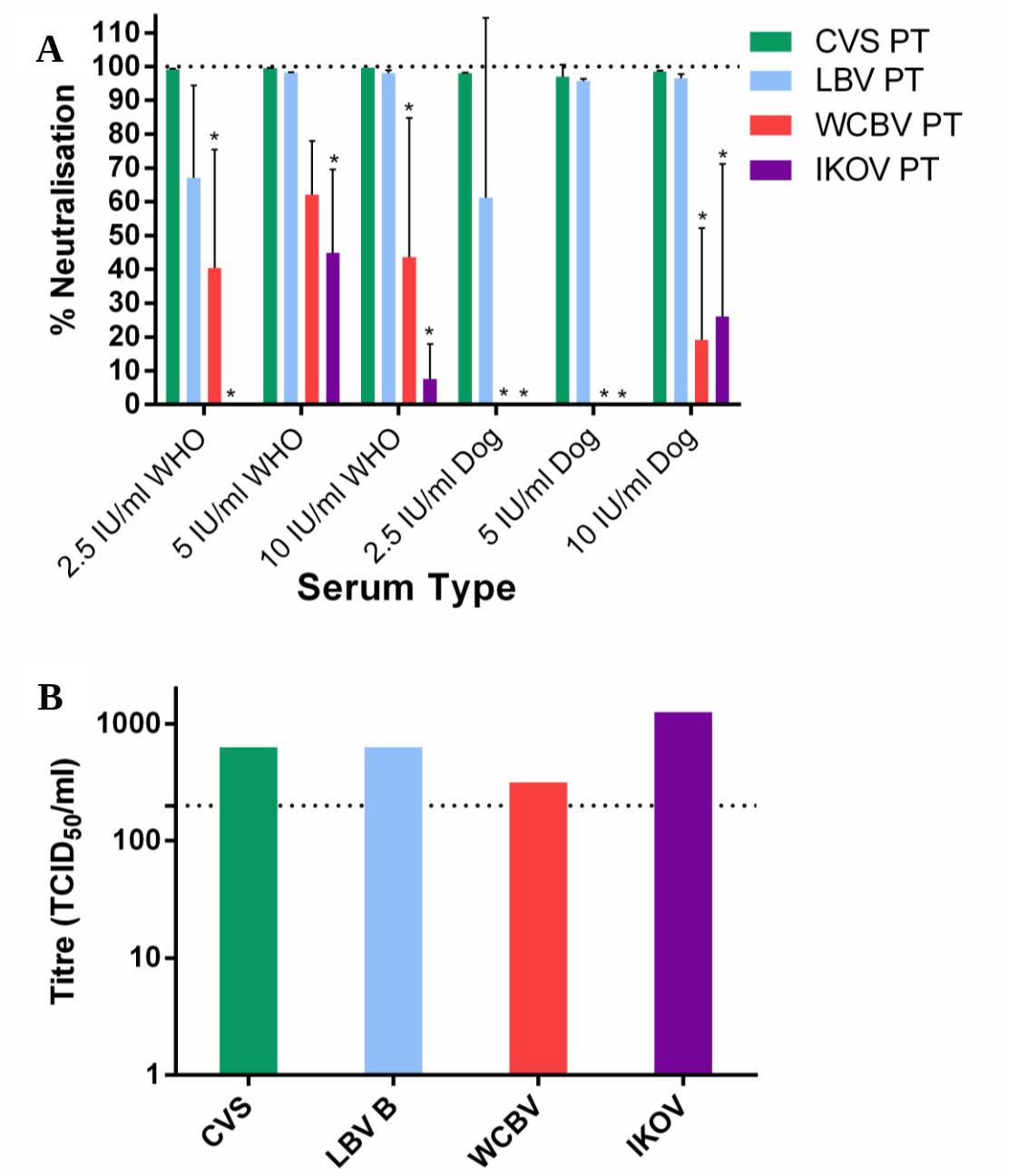


Figure 4.5: Neutralisation profile of lyssavirus PTs against increasing concentrations of hyperimmune sera. (A) PT neutralisation assay. WHO serum was obtained as an international reference standard from NIBSC and the dog serum was created from a pool of hyperimmune dog sera. A 2-way ANOVA was used to determine any significant differences to neutralisation of CVS by each serum. Differences are indicated by asterisks; p*≤0.05. (B) Back titrations of each PT used in the assay. The dotted line indicates the target titre following PT dilution. Error bars represent standard deviation (SD) about the mean of three replicate in-assay neutralisation tests.

of neutralisation was evident with WCBV neutralised by 19% and IKOV by 26% respectively. These results suggest that there may be a significant degree of cross neutralisation between phylogroups I and II in cases of high antibody titre post vaccination, however there is no cross neutralisation of phylogroup III or IKOV by the RABV hyperimmune sera.

The degree of cross neutralisation between phylogroups using high titre vaccinee serum specific to RABV had been investigated so next the degree of cross neutralisation when applying the serological threshold for protection against RABV was investigated alongside a ten-fold dilution of sera as described in Figure 4.1 and Figure 4.3 for phylogroup I PTs. The same panel of four PTs used in Figure 4.5 were run against serum specific to each of the PTs: WHO and VLA gold standard reference sera specific to RABV and rabbit serum raised against LBV B, WCBV and IKOV respectively (Figure 4.6).

A 2-way ANOVA test was used to compare the degree of neutralisation of each PT to the percentage neutralisation of PT by its homologous serum i.e. for the LBV specific serum, the neutralisation of each PT was compared to neutralisation of LBV. Values significantly different to the homologous PT neutralisation are indicated by an asterisk. An alpha level of 0.05 was used for each comparison.

At both 0.05 IU and 0.5 IU WHO serum was unable to neutralise LBV B, WCBV or IKOV to a similar degree as CVS whereas the VLA serum was able to neutralise WCBV to a statistically similar degree as CVS at both 0.05 IU and 0.5 IU. The LBV B serum was unable to neutralise any PT substantially at 0.05 IU, even LBV B however at 0.5 IU

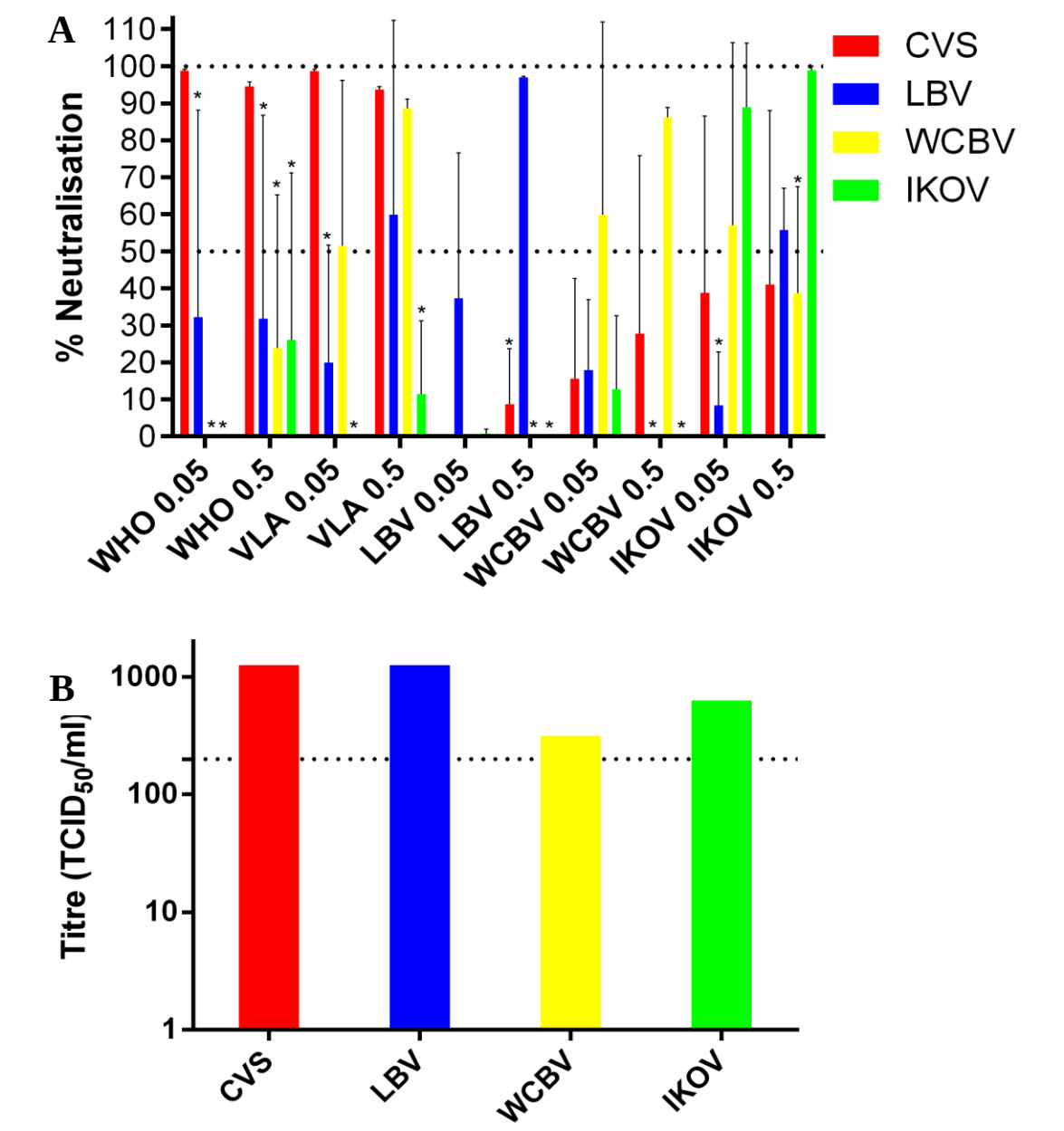


Figure 4.6: Cross neutralisation between the lyssavirus phylogroups. (A) PT neutralisation assay. Each PT was run against a panel of specific sera at a concentration of either 0.5 IU/ml or 0.05 IU/ml. A two way ANOVA was used to determine significant differences between each PT and the homologous PT versus serum. Significance is indicated by an asterisk; *$p \leq 0.05$. (B) Back titrations of each PT used in the assay. The dotted line indicates the target titre following PT dilution. Error bars represent standard deviation (SD) about the mean of three replicate in-assay neutralisation tests.

it specifically and completely neutralised LBV B only. The WCBV specific serum showed a similar profile to the LBV B serum, only being able to significantly neutralise WCBV at both concentrations whereas the IKOV specific serum was able to neutralise both CVS and WCBV to a similar extent to IKOV at 0.05 IU. At 0.5 IU whilst CVS was still significantly neutralised, WCBV was no longer significantly neutralised but a substantial level of neutralisation was maintained and LBV B was also significantly neutralised. This suggests that below or at the recognised cut-off for protection of 0.5 IU/ml vaccine induced sera (WHO and VLA) are unable to neutralise any PTs outside of phylogroup I. In contrast IKOV specific serum appears able to neutralise PTs from all three phylogroups at 0.05 IU and 0.5 IU, despite being the most divergent lyssavirus identified to date.

4.6 Discussion

The pseudotype neutralisation assays in this chapter have identified a strong level of intra-phylogroup neutralisation within phylogroups I and II. The results have also suggested a degree of cross neutralisation between IKOV and WCBV though the reciprocal reactions showed little to no cross neutralisation. Interestingly, higher concentrations of hyperimmune sera demonstrated some cross neutralisation between phylogroups I and II.

One element of the data produced by the PNAs in this Chapter is the considerable degree of variation between replicates. Every neutralisation was conducted in triplicate however the variation in level of luminescence differed substantially between some replicates. This is likely a result of the mechanism by which luminescence is detected. Certainly, the standard virus neutralisation assays performed when assessing serological status in

samples depend on the detection of virus antigen in a sample where antibodies in the test sample are unable to neutralise input virus. This WHO and OIE Gold standard serological test then relies on reader interpretation of the presence or absence of antigen following fixation and staining of the monolayer with a FITC conjugated polyclonal antibody preparation directed against the virus N protein. As a result the readout of these assays is simply the reciprocal titre at which neutralisation is observed in comparison to control sera. In contrast, using the pseudotype neutralisation assay, a measure of luminescence is taken following incubation with or without test sera, which by virtue of assays dependent on chemiluminescence, will exhibit a much higher degree of variability across replicates. As such the PNA can be performed using alternative reporter genes such as GFP or β-galactosidase but in this study efforts were made to quantify neutralisation. This variation in RLU between replicates has been found in a number of other laboratories using this assay system (Dr. Wright University of Westminster and Dr Both St George's University – personal communication) which suggests that there is an inherent issue with the luciferase read out of PNAs. This variation however is measured and recorded and the advantages of an immediate quantitative read out of each well outweigh the disadvantages of the variation observed. A study into the reduction of variation between replicates would aid further studies of this nature.

In addition to the variation between replicates, the variable back titres of PTs within each assay must also be considered. Although efforts were made to ensure a back titre of 200 $TCID_{50}$/ml, this was not always achieved and therefore there was a variation in PT titre between PTs within assays. This may have affected the neutralisation profiles of PTs as a lower than 200 $TCID_{50}$/ml input could result in more substantial neutralisation

than would have resulted had the back titration been closer to 200 $TCID_{50}$/ml and vice versa.

A very strong degree of cross neutralisation within phylogroup I at both 0.5 IU and 0.1U was confirmed by PNAs in Figures 4.1 and 4.2. This data is supported by current knowledge of phylogroup I viruses however studies have shown varying levels of cross neutralisation within phylogroup I, for example the HDCV was able to protect more efficiently against CVS and EBLV-2 infection than ABLV or EBLV-1 (Brookes *et al.*, 2005). In contrast antigenic cartography has shown that ABLV and KHUV appear antigenically identical to the RABVs. IRKV, ARAV and EBLV-1 were next most similar to RABV followed finally by EBLV-2 (Horton *et al.*, 2010). These contrasting studies suggest that the picture of the antigenicity of phylogroup I lyssaviruses is still not well defined however the data generated in this investigation provide some insight into the utility of the PNA to investigate further the field of cross neutralisation between lyssaviruses.

The assay involving the phylogroup I PT panel against sera from divergent lyssaviruses shows again the difference between glycoproteins in phylogroup I and appeared to suggest that WCBV and IKOV specific serum were able to neutralise some phylogroup I PTs to a small extent. This pattern of neutralisation may be a result of the sera used. The sera were obtained from rabbits inoculated with inactivated PT preparations (see Section 5.5) meaning that the sera were polyclonal. This may result in the targeting of a variety of areas along the glycoprotein, some of which may be well conserved among all lyssaviruses. Although the antigenic sites along the lyssavirus glycoprotein have been well defined and do differ between phylogroups, the lack of a crystal structure means

that immunogenic domains which rely on the conformation of the protein may not be so well understood and it is possible that domains like this are targeted by some antibodies in these polyclonal serum preparations. Indeed it is likely that some neutralising antibodies are targeted to less divergent domains on the ectodomain of G although until an atomic structure for G is generated such areas are difficult to predict.

Following confirmation of cross neutralisation within PTs representing lyssaviruses from phylogroup I it was necessary to determine whether there is a strong level of cross neutralisation between the members of phylogroup II. PTs representing all viruses in phylogroup II were generated, including representatives of three of the four LBV lineages. Unfortunately it was not possible to generate sufficient titre for LBV lineage D and as such this glycoprotein was excluded from the study. However, with only a single isolate of LBV lineage D having been detected evaluation of cross neutralisation between the viruses for which PTs were generated is sufficient to gauge the degree of cross neutralisation within the phylogroup. There was clear cross neutralisation between all members of phylogroup II (Figure 4.4) as well as an unexpectedly high degree of cross neutralisation of WCBV and IKOV PTs by the LBV lineage B specific serum. The presence of a strong intra-phylogroup neutralising response, within both phylogroups I and II has been identified previously (Badrane *et al.*, 2001) so the data in this PNA confirms this and extends the existing data to encompass all but one of the currently characterised viruses that are grouped within these phylogroups. Furthermore this study has given proof of concept for the PNA as an alternative technique for determining cross neutralisation as previous studies have been limited to quantifying neutralising responses to plasmids containing the phylogroup II glycoproteins as opposed to live virus. These previous studies utilised plasmid DNA vectors expressing PV or MOKV glycoprotein

sequences under the control of a cytomegalovirus promoter (CMV). These DNA preparations were then purified and injected intramuscularly into mice. Although specific immune responses were raised to the glycoprotein DNA sequences in these plasmids, the antibody responses were characterised by ELISA (Badrane *et al.*, 2001; Bahloul *et al.*, 1998). This limits the ability of the study to predict neutralisation as the ELISA used was only able to detect binding antibody, it was unable to identify neutralising antibody. The PNA used here is able both to identify neutralising antibodies and to generate anti-sera against the glycoprotein in a more natural conformation; expressed on the surface of a PT particle which may induce antibodies more similar to those that might be generated as a result of lyssavirus infection.

The confirmation of this intra-phylogroup cross neutralisation is essential to the remainder of this investigation as it means that a construct containing the antigenic epitopes of any phylogroup II lyssavirus should be sufficient to neutralise any other phylogroup II virus. According to the results in Figure 4.4 it may also be sufficient for a small degree of neutralisation of WCBV or IKOV. This suggests that if a pan-lyssavirus cross protective candidate was to be developed it would have to contain antigenic features from all three phylogroups and perhaps IKOV as well in order to produce strongly neutralising antibodies against all currently characterised lyssaviruses.

The further investigations into cross neutralisation between all three phylogroups confirmed that antibodies induced by vaccination would provide no protection against infection with phylogroup II or III viruses, even at concentrations 20 times greater than those which neutralise phylogroup I constructs (Hanlon *et al.*, 2005; Horton *et al.*, 2014). The possibility however of some cross neutralisation between IKOV and WCBV

however is interesting. Currently the classification of IKOV into a phylogroup is ongoing however the data from this experiment suggest that it would be best placed in a new phylogroup; phylogroup IV. This is due to the lack of a strong neutralising response between WCBV and IKOV, unlike the strength of the neutralising responses within the other two phylogroups. Despite this, there did appear to be a significant degree of cross neutralisation of CVS, LBV B and WCBV by the IKOV specific sera in Figure 4.6. The basis for this result is unknown as there have been no published investigations into cross neutralisation of IKOV with other lyssaviruses however both IKOV and LBV B are African lyssaviruses and there is evidence that WCBV may also be circulating in Africa (Kuzmin *et al.*, 2008a) so there may be some antigenic feature which all African lyssaviruses share.

The overall finding is that there is strong intra-phylogroup neutralisation but there is little inter-phylogroup neutralisation at 0.5 IU/ml. It would be valuable to investigate cross neutralisation between phylogroups using sera from different species sources and also directed against different vaccine or RABV strains as there may be differences between the immune responses to different vaccine strains, for example it was found that an animal vaccine; Rabiffa was able to protect dogs against challenge with LBV, whereas the HDCV (Imovax) was unable to protect against LBV challenge but was able to protect against CVS and DUVV challenge (Fekadu *et al.*, 1988). In addition, the cross neutralising capacity of IKOV serum could be further investigated by increasing the concentration of serum against a panel of PTs in order to determine whether increasing antibody concentration improves its cross neutralising capacity.

Chapter 5: Investigating the effect of mutation to antigenic sites within the lyssavirus glycoprotein on serological neutralisation

5.1 Introduction

An extensive panel of lyssavirus pseudotypes (PT) was generated (Chapter 3) that encompassed lyssavirus glycoproteins representative of both wildtype and mutated glycoprotein sequences. As a tool to investigate the difference in neutralisation profiles of different sera for the lyssavirus glycoprotein a series of antigenic site swap mutant glycoproteins were developed containing single and multiple antigenic site swaps between a representative rabies isolate (CVS) (phylogroup I) and Lagos Bat Virus (lineage B) (phylogroup II). These isolates were chosen due to the availability of live virus, glycoprotein sequence and potently neutralising hyperimmune sera specific for each. A panel of single and multiple site swaps was then investigated to evaluate any potential role for the different antigenic sites in neutralisation. The antigenic site swap mutant PT viruses were assayed against sera specific to both classic rabies virus as well as LBV to identify any potentially cross neutralising constructs whilst the panel of wildtype PTs comprising phylogroups II and III (described in Chapter 4) was assayed against a range of sera from different phylogroups in order to identify any existing cross neutralisation between and within divergent phylogroups. Alongside this, sera specific for mutated glycoproteins was generated in vivo to enable an assessment of any alteration in antigenicity seen following mutation.

5.2 Assessment of the CVS PT Panel

The first panel of PTs to be assessed by PNA were the CVS to LBV single and full antigenic site swaps. The titres of each of the PTs generated in this panel are shown in

Figure 5.1. The titres of the CVS panel range from CVS to LBV IIaIIb with the lowest titre of $10^{3.26}$ $TCID_{50}$/ml to CVS to LBV IIa with the highest titre of $10^{7.30}$ $TCID_{50}$/ml. There was an approximately 4 log difference between the lowest and highest titres however all titres were sufficiently high to enable reliable dilution for input into the PNA. As described in Chapter 3 the CVSFSS PT could not be generated to a sufficient titre to test (Section 3.6).

Each of the PTs in the CVS panel were run against three different sera. WHO serum was obtained from NIBSC and is utilised as the WHO gold standard reference for vaccinated humans in current diagnostic tests. VLA serum was obtained from a panel of vaccinated dogs and was used as the OIE gold standard reference in diagnostic tests. Both of these sera are specific to classic rabies virus in phylogroup I as they were raised against vaccine strain RABVs. The third serum utilised was obtained from a rabbit experimentally inoculated with β-propiolactone inactivated LBV lineage B PT virus (see Section 5.5). As described in Section 2.3.3, three different sera were used to assess the neutralisation of the panels of different wildtype and mutated PTs. Each of the three sera were diluted to 0.5 IU/ml, following the established dilution series utilised by the FAVN, and 50µl of these serum dilutions were exposed to 50µl of diluted PT in triplicate. A reduction in the level of luminescence was detected in cases of PT neutralisation by the test sera and the percentage neutralisation of each PT by each serum was calculated according to the value for luminescence generated by the untreated PT (Figure 5.2). The back titrations in Figure 5.2 (B) confirm that all PT input titres were close to the target of 200 $TCID_{50}$/ml so all resulting neutralisation data should be reliably comparable. All CVS based PTs including the CVS wildtype PT were almost completely neutralised by both RABV specific sera; WHO and VLA which suggests that

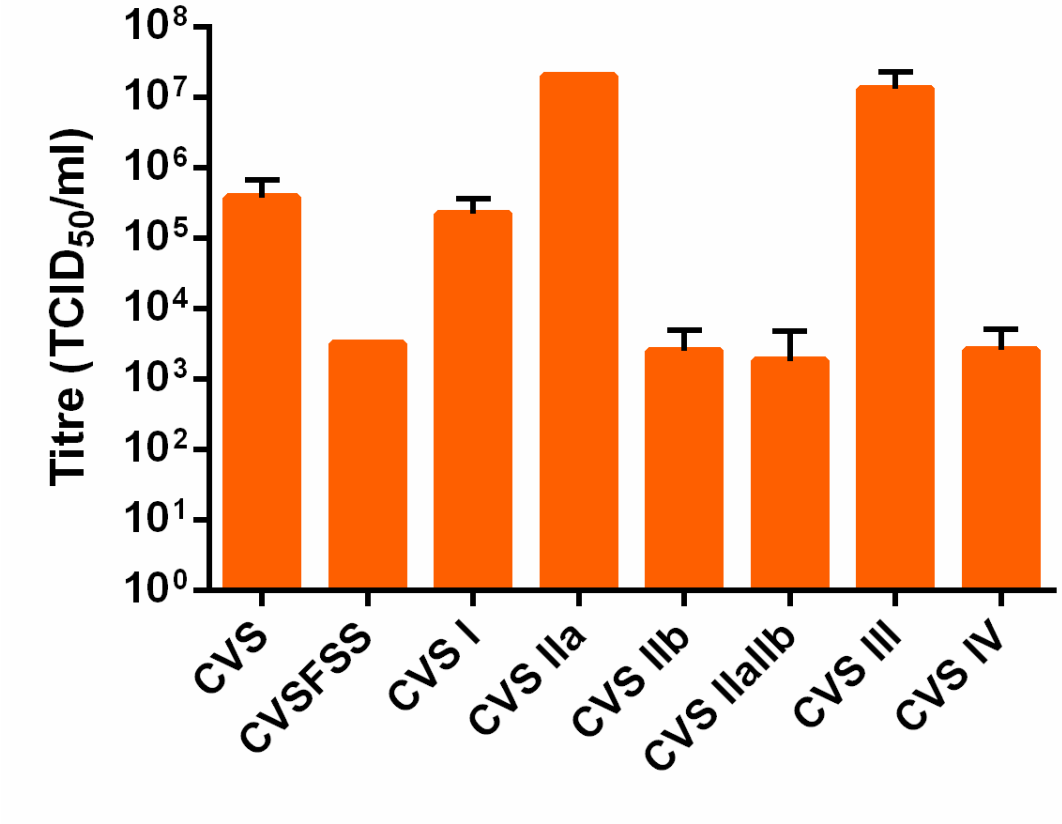


Figure 5.1: The titres of the panel of wildtype, single and full antigenic site swaps between CVS and LBV. Titre was measured in $TCID_{50}$/ml as determined on BHK-21 cells. Error bars represent standard deviation (SD) about the mean of three replicate in-assay titrations.

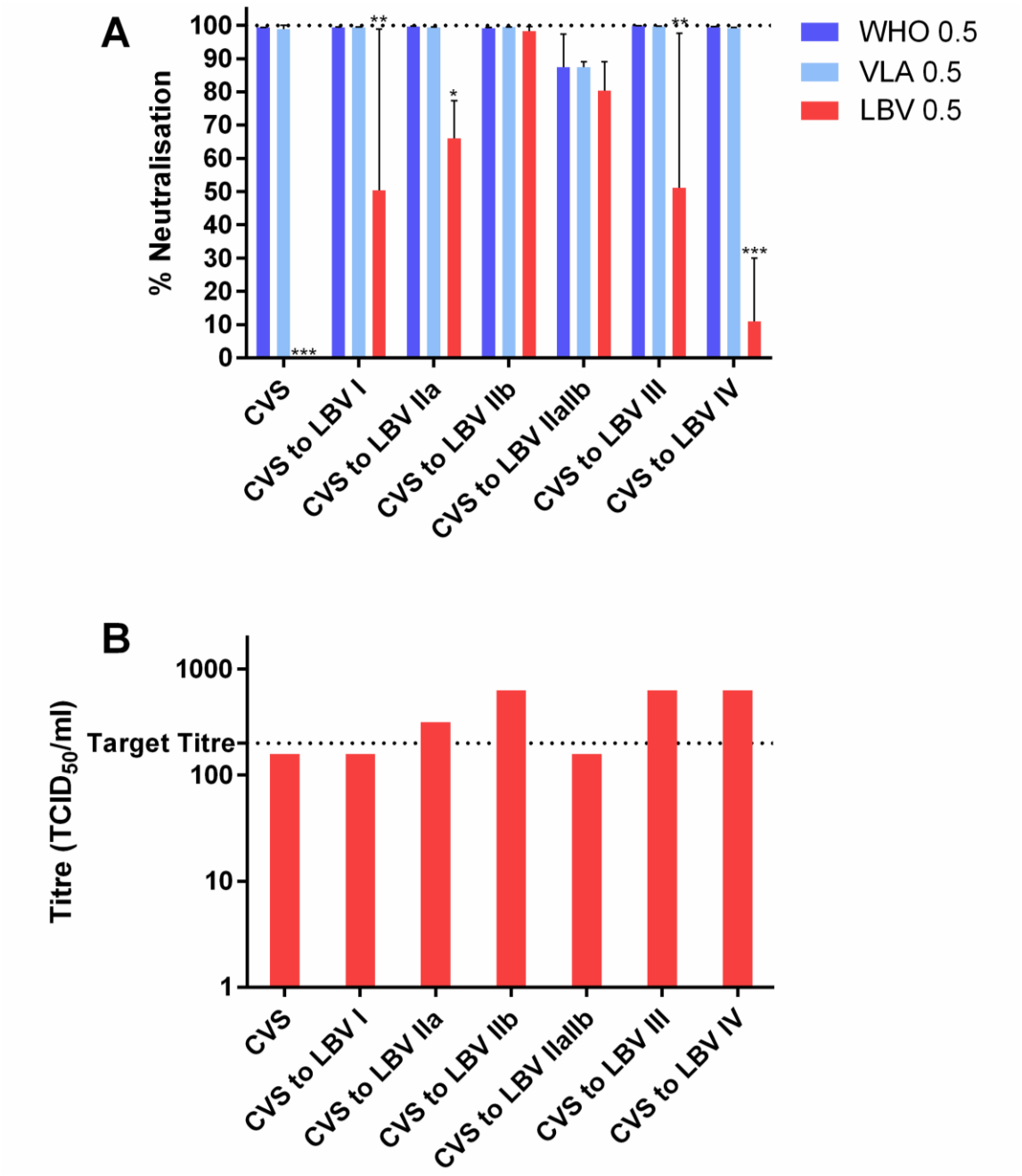


Figure 5.2: CVS antigenic site swap PT neutralisation profiles. (A) The result of the CVS panel of antigenic site swap mutants PNA against both phylogroup I specific sera, WHO and VLA as well as phylogroup II specific sera, LBV. All sera were diluted to 0.5 IU/ml. A two way ANOVA was used to determine significant differences between neutralisation of each PT and neutralisation of CVS by its homologous WHO and VLA sera. Significance is indicated by an asterisk.; *p≤0.05, **p≤0.005, ***p≤0.001. (B) Back titrations of each PT used in the assay. The dotted line indicates the target titre following PT dilution. Error bars represent standard deviation (SD) about the mean of three replicate in-assay neutralisation tests.

no single antigenic site swap to that of LBV within the CVS G was sufficient to affect phylogroup I neutralisation. The CVS wildtype PT was not neutralised by the LBV B serum which confirms a lack of cross neutralisation between these glycoproteins, however a number of single and multiple antigenic site swap mutants were neutralised by the LBV B specific sera, with those mutants containing LBV site IIb residues being neutralised to a significantly similar extent as CVS wt. CVS to LBV IIb was most strongly neutralised by 98% followed by CVS to LBV IIaIIb by 80%. CVS to LBV IIa was neutralised by 65% however this was significantly different to CVS wt neutralisation. This suggests that antigenic site II may play an important role in neutralisation of phylogroup II viruses though CVS to LBV I and III were also neutralised by 50% and 51% respectively which may suggest that these major antigenic sites may also contribute to neutralisation, though not to a significant extent.

The CVS to LBV IV mutant was very strongly neutralised by both the WHO and VLA serum whilst being neutralised by only 11% by the LBV B serum. This may suggest that site IV plays only a very minor role in neutralisation of phylogroup II viruses as its neutralisation profile by the three test sera was very similar to that of CVS wildtype.

Despite numerous attempts with the CVSFSS PT, back titration of virus did not yield sufficient virus to indicate that neutralisation was occurring. As such, attempts to study the effects of the addition of different sera to the CVSFSS PT were not performed.

5.3 Assessment of the LBV PT Panel

Having established the neutralisation profile of the panel of CVS mutants against a range of phylogroup I and II specific sera, the reciprocal PTs in the LBV to CVS site swap

panel were run against the same panel of sera in an exact experimental repeat. The titres of the panel of LBV site swap mutants are shown in Figure 5.3 and the result of the PNA is shown in Figure 5.4. The titres of the LBV panel of PTs were more consistent than the reciprocal CVS PT panel. All titres were approximately 10^5 $TCID_{50}$/ml except LBV to CVS III which had a lower titre of $10^{3.18}$ $TCID_{50}$/ml. Each of these PTs was diluted to an endpoint titre of 200 $TCID_{50}$/ml for input into the PNA (Figure 5.4).

The back titration in Figure 5.4 B shows that all PT dilutions were successful as all back titres were close to 200 $TCID_{50}$/ml. In Figure 5.4 A the results of the PNA show a considerable amount of variation for many of the values, overall there seems to be a greater amount of variation in the LBV PNA than the CVS PNA. Despite this variation there was a clear pattern in the neutralisation profiles observed. The LBV wildtype PT was neutralised by the LBV specific sera, as expected however, interestingly the LBV wildtype PT was also very strongly neutralised by both of the RABV specific sera which contrasts with the CVS wildtype PT result which was not neutralised at all by the LBV specific sera. In addition the LBVFSS PT which contains all the antigenic epitopes of CVS was also strongly neutralised by both the RABV and LBV specific sera which may suggest that there are important neutralising epitopes outside of the defined antigenic sites for neutralisation of LBV and that these epitopes may be cross neutralising for RABV. The rest of the panel of single antigenic site swap mutants were all also strongly neutralised by the LBV specific sera which supports the hypothesis of the importance of epitopes outside of the antigenic sites. Those antigenic sites which do, however appear to play a role in phylogroup I neutralisation were indicated by PTs which were more strongly neutralised by the RABV specific sera than the LBV sera.

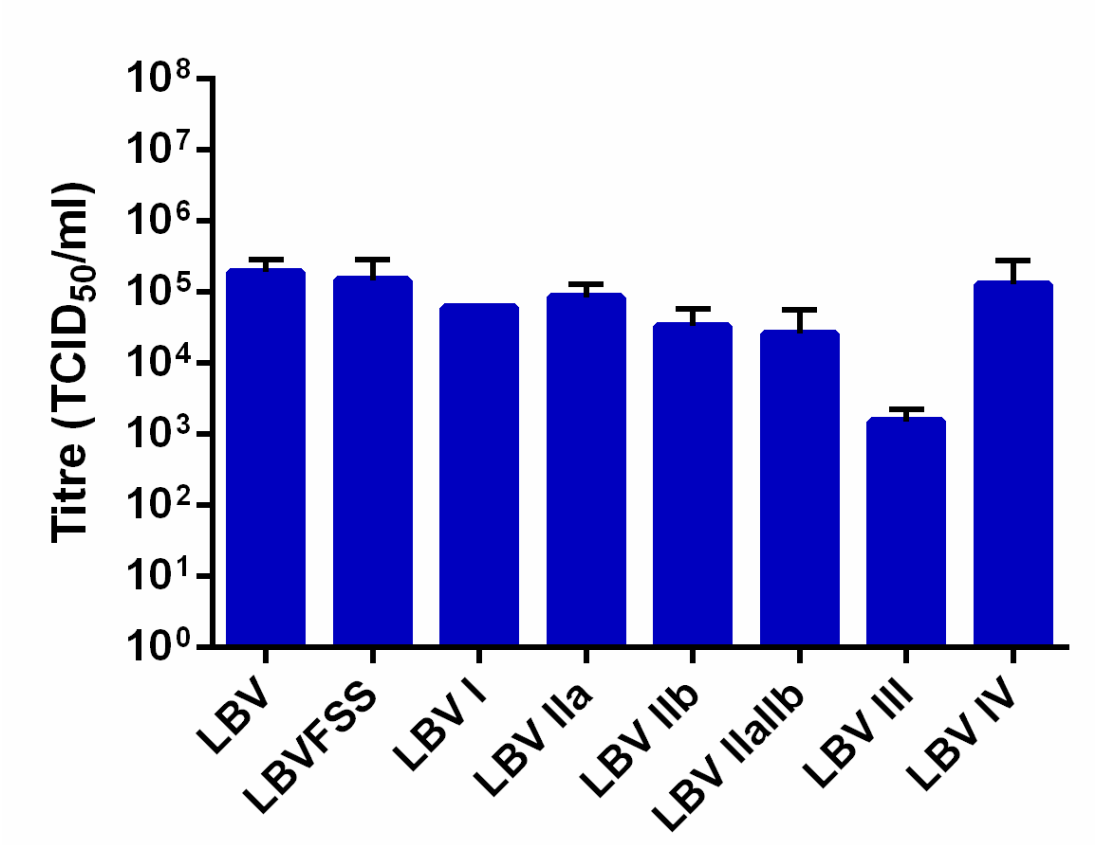


Figure 5.3: The titres of the panel of wildtype, single and full antigenic site swaps between LBV and CVS. Titre is measured in $TCID_{50}$/ml as determined on BHK-21 cells. Error bars represent standard deviation (SD) about the mean of three replicate in-assay titrations.

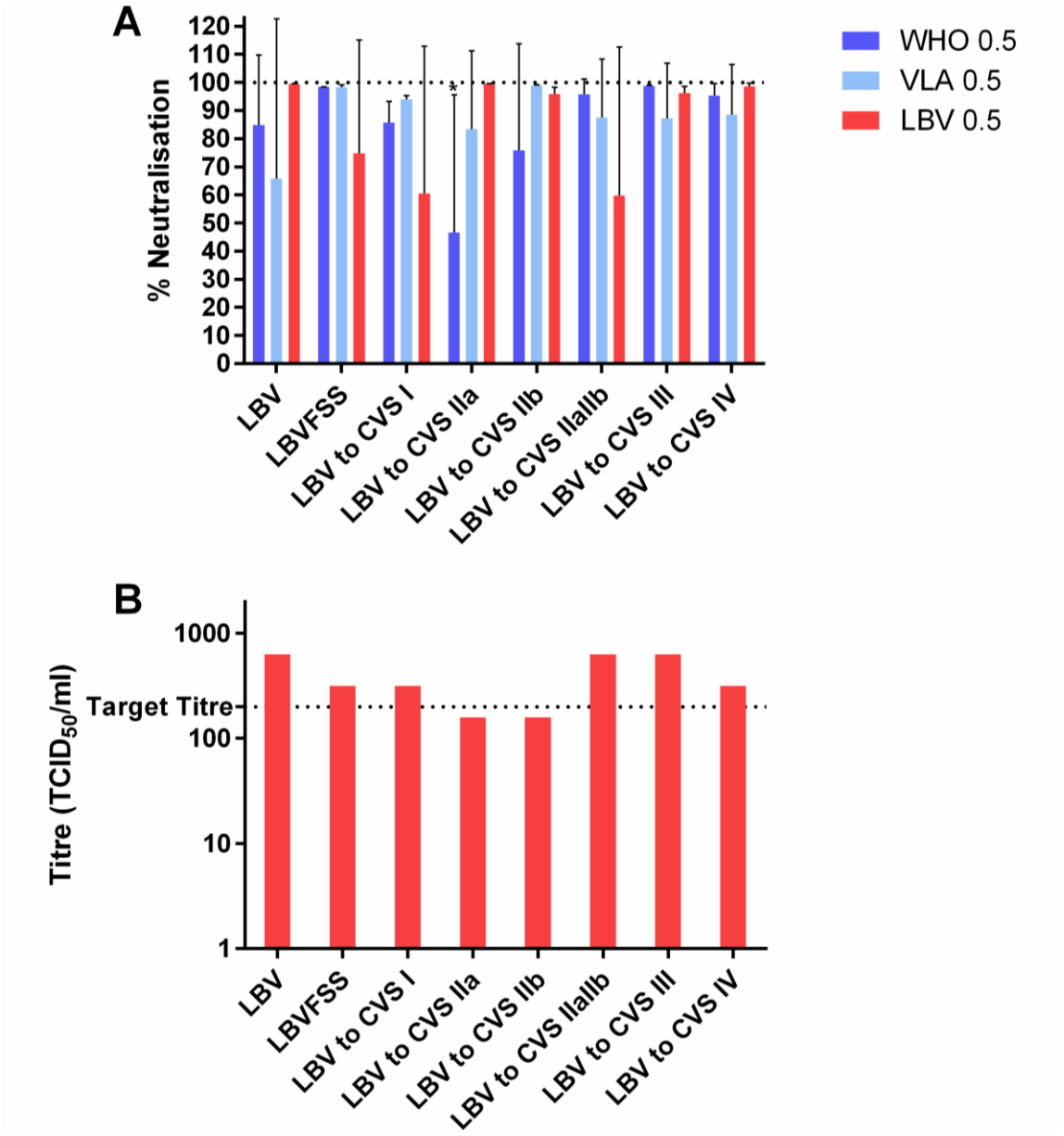


Figure 5.4: LBV antigenic site swap PT neutralisation profiles (A) The result of the LBV panel of antigenic site swap mutants PNA against both phylogroup I specific sera, WHO and VLA as well as phylogroup II specific sera, LBV. All sera were diluted to 0.5 IU/ml. A two way ANOVA was used to determine significant differences between neutralisation of each PT and neutralisation of LBV by its homologous LBV serum. Significance is indicated by an asterisk.; *$p \leq 0.05$. (B) Back titrations of each PT used in the assay. The dotted line indicates the target titre following PT dilution. Error bars represent standard deviation (SD) about the mean of three replicate in-assay neutralisation tests.

From Figure 5.4 these PTs appear to be LBV to CVS sites I, IIb, IIaIIb and III. LBV to CVS site I and LBV to CVS IIb were neutralised by all three sera by over 99% suggesting both phylogroup I and II neutralising epitopes were present. LBV to CVS IIaIIb was neutralised by WHO and VLA sera by 86% and 98%, respectively and 91% by the LBV specific sera. The LBV to CVS III PT was neutralised by the WHO and VLA sera by 97% and 99% respectively and the LBV sera by 99%, again suggesting the importance of site III in a phylogroup I neutralising response but only neutralisation of LBV to CVS IIa by WHO serum was significantly different to LBV wt neutralisation.

The pattern of epitopes identified as holding an important role in phylogroup I neutralisation (Figure 5.4) was similar to that identified for phylogroup II neutralisation (Figure 5.2). The important antigenic sites appear to include site II (the combination of sites IIa and IIb), site I and site III. These epitopes when swapped alone, and also in combination with all others appeared to affect the neutralisation profile of the resulting PTs.

The finding that LBV PT was neutralised by WHO and VLA sera by 85% and 66%, respectively, was unexpected, based on the lack of neutralisation of LBV virus by RABV specific sera. This suggests the possibility that the density of G protein on the surface of PT particles may affect the ability of sera to neutralise the PT. In order to investigate whether this is the case a PNA was set up using a 1 in 10 dilution of the sera from the PNAs in Figures 5.2 and 5.4. The result of this assay can be seen in Figure 5.5. The assay in Figure 5.5(A) shows CVS and LBV PTs versus the RABV and LBV specific sera at a concentration of 0.5 IU/ml. The CVS PT was strongly neutralised by both RABV specific sera whilst only 34% neutralised by the LBV specific sera. The

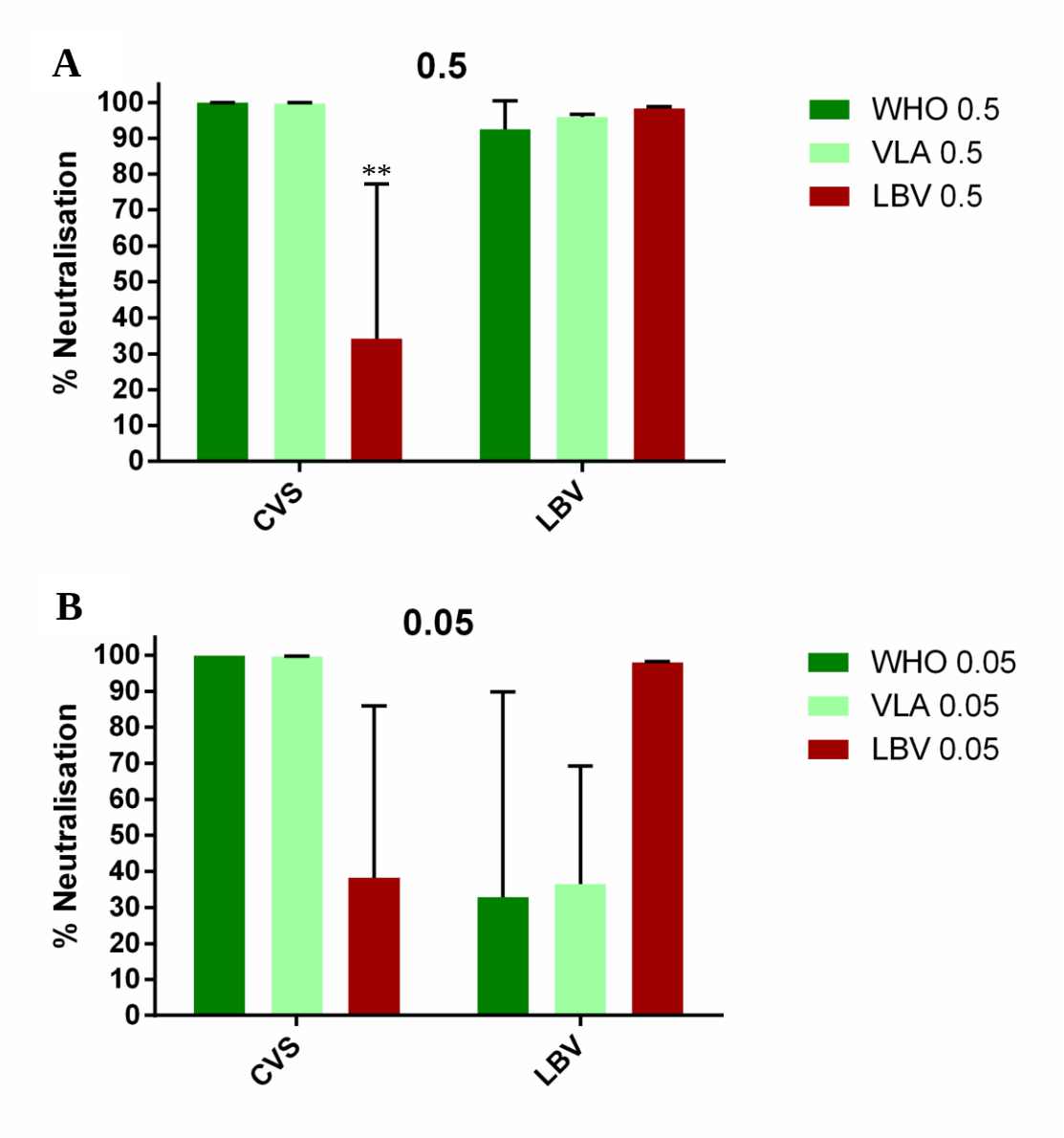


Figure 5.5: Assessment of the effect on serum dilution and the neutralisation profiles of PTs (A) CVS and LBV PTs against the RABV specific WHO and VLA sera and the LBV specific sera at 0.5 IU/ml. (B) CVS and LBV PTs against the RABV specific WHO and VLA sera and the LBV specific sera at 0.05 IU/ml. Error bars represent standard deviation (SD) about the mean of three replicate in-assay titrations. A two way ANOVA was used to determine significant differences between neutralisation of each PT by each serum and neutralisation of each PT by its homologous serum. Significance is indicated by an asterisk; **$p \leq 0.005$.

neutralisation profile of the LBV PT again demonstrated neutralisation by each of the WHO, VLA and LBV sera by 92%, 96% and 98%, respectively. In Figure 5.4 (B) both PTs were run against sera at 0.05 IU/ml and the neutralisation profiles of both CVS and LBV PTs reflected the neutralisation profiles seen with the FAVN with the CVS PT neutralisation profile matching that of the 0.5 IU/ml assay while the LBV PT was neutralised by the WHO and VLA sera by only 33% and 37% respectively whilst being 98% neutralised by the LBV specific serum however neutralisation of CVS was not found to be significantly different to neutralisation of LBV at 0.05 IU/ml.

The results of this assay suggest that there may be less glycoprotein present on the surface of the LBV PTs than is present on naturally produced virions and may also be possible that there is less G on the surface of the CVS PT due to the expected neutralisation profile of LBV only being achieved at a 1 in 10 dilution of the reference sera. As a result of this finding, the LBV PNA was repeated with the sera at a concentration of 0.05 IU/ml (Figure 5.6). The result of the LBV PNA against 0.05 IU/ml of each serum showed a clearer neutralisation profile than the 0.5 IU/ml PNA (shown in Figure 5.4). In Figure 5.6(A) the LBV wildtype PT was strongly neutralised by the LBV specific serum whilst being poorly neutralised by the WHO serum and not neutralised at all by the VLA serum. If the pattern of neutralisation by RABV specific sera verses LBV specific sera is used to suggest which antigenic sites may play dominant roles in neutralisation, three PTs appeared to affect neutralisation more significantly: LBVFSS, LBV to CVS I and LBV to CVS III. These three PTs were neutralised to a greater degree by the RABV specific sera than the LBV specific sera which suggests that sites I and III play an important role in phylogroup I neutralisation however these neutralisation profiles were not significantly different to wt LBV. This selection of sites was similar to

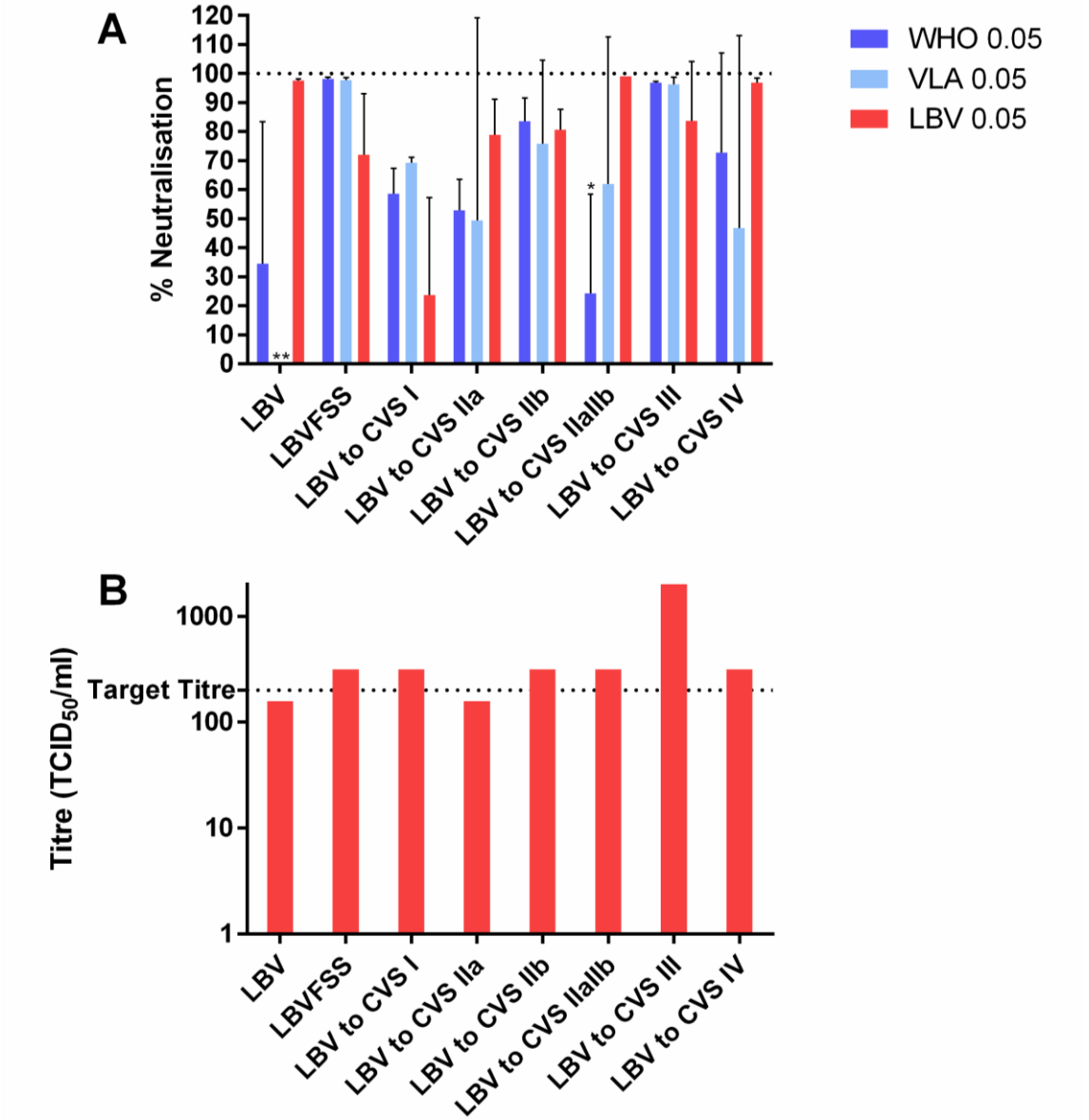


Figure 5.6: LBV PNA versus sera at 0.05 IU/ml. (A) The result of the LBV panel of antigenic site swap mutants PNA against both phylogroup I specific sera, WHO and VLA as well as phylogroup II specific sera, LBV. All sera were diluted to 0.05 IU/ml. A two way ANOVA was used to determine significant differences between neutralisation of each PT and neutralisation of LBV by its homologous LBV serum. Significance is indicated by an asterisk.; *$p \leq 0.05$, **$p \leq 0.05$. (B) Back titrations of each PT used in the assay. The dotted line indicates the target titre following PT dilution. Error bars represent standard deviation (SD) about the mean of three replicate in-assay neutralisation tests.

that identified in the previous 0.5 IU/ml assay in Figure 5.4.

5.4 Investigation of cross neutralisation between phylogroups

Chapter 4 demonstrated that within each phylogroup there was considerable cross neutralisation but that between phylogroups the ability of sera to neutralise more divergent viruses varied. The next area of interest was to investigate the ability of different sera, specific for individual lyssaviruses, to neutralise a panel of lyssaviruses. To this end, PT virus was inoculated into rabbits to generate specific neutralising sera against viruses for which no sera were available.

A range of ten PTs were selected for inoculation into rabbits based on the availability of specific sera (Section 2.9.1). Each of the rabbit derived specific sera were then tested against a panel of lyssavirus PTs to assess the potential for cross neutralisation between viruses.

Initially, prior to inoculation, the titres of the PTs inoculated into the rabbits were determined (Figure 5.7). All PTs were generated to a high titre ($>10^6$ $TCID_{50}$/ml) with the exception of PTs for WCBV and IKOV although for both titres were still $>10^4$ $TCID_{50}$/ml. Once the sera had been generated, each sample was run against its homologous PT in order to determine the titre of specific antibodies. The titres of each serum sample are shown in Table 5.1.

The sera with the highest tires were CVS to LBV I and LBV lineage A whilst the lowest titres were LBV to CVS IIaIIb, LBV lineage B and IKOV. The reason for the variation

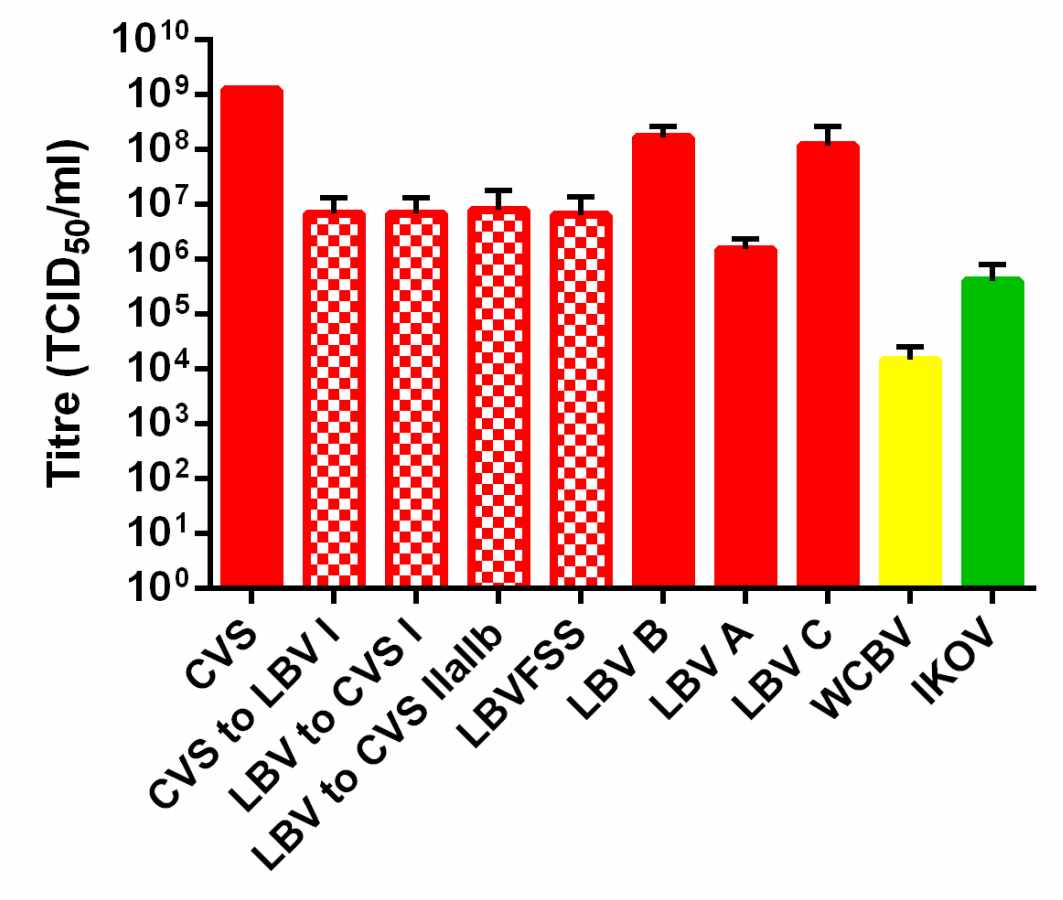


Figure 5.7: Titres of PTs inoculated into rabbits to produce specific polyclonal sera. Solid colour bars represent wildtype glycoprotein PTs and chequered bars represent antigenic site mutants. Error bars represent standard deviation (SD) about the mean of three replicate in-assay titrations.

Table 5.1: Serum titres of each rabbit polyclonal serum generated against a range of inactivated PT viruses. Titres are given both in International Units per ml and reciprocal titre i.e. 50% end point.

Rabbit Sera	Titre (IU/ml)	Reciprocal Titre
CVS	512.0	40959.9
CVS to LBV I	2048.0	163839.6
LBV to CVS I	362.0	28962.9
LBV to CVS IIaIIb	16.0	1280.0
LBVFSS	1448.2	115851.4
LBV A	2896.3	231702.4
LBV B	8.0	640.0
LBV C	362.0	28962.9
WCBV	90.5	7240.7
IKOV	22.6	1810.2

between the serum titres was unknown so the protein concentration of each inocula (inactivated PT prep) was determined via spectrophotometer and absorbance at the 280 nm wavelength. A standard curve was constructed using a series of known concentrations of BSA in PBS and the resulting curve was then used to approximate the protein concentration of each of the inocula. The standard curve generated is shown in Figure 5.8. The concentration of each inoculum was determined from the curve and the results are displayed in Table 5.2.

The protein concentrations of all inocula were similar with the lowest being WCBV at 3.66 mg/ml and the highest being CVS at 5.11 mg/ml. In order to determine which factor had the greatest influence on serum titre, statistical analyses were used to compare the serum titre values with both the PT titre and the protein concentration of each inocula. An alpha level of 0.05 was used for all statistical tests. The serum titre did not correlate with PT titre $r(10) = -0.04359$, $p = 0.9048$ and although there was a slightly stronger positive correlation of serum titre with inoculum protein concentration, this relationship remained insignificant $r(10) = 0.3265$, $p = 0.3571$. The variation in serum concentrations following inoculation was therefore likely related to animal to animal variation.

Once the serum concentration of each sample had been determined using the PNA a confirmation of each serum titre was conducted via FAVN. A function of the FAVN test is that only CVS virus is used which means that any sera generated against divergent lyssavirus PTs may not cause neutralisation of the phylogroup I CVS virus. Despite this, each rabbit derived serum was run against CVS PT on a standard PNA and against live CVS virus on a standard FAVN assay in order to identify any significant differences

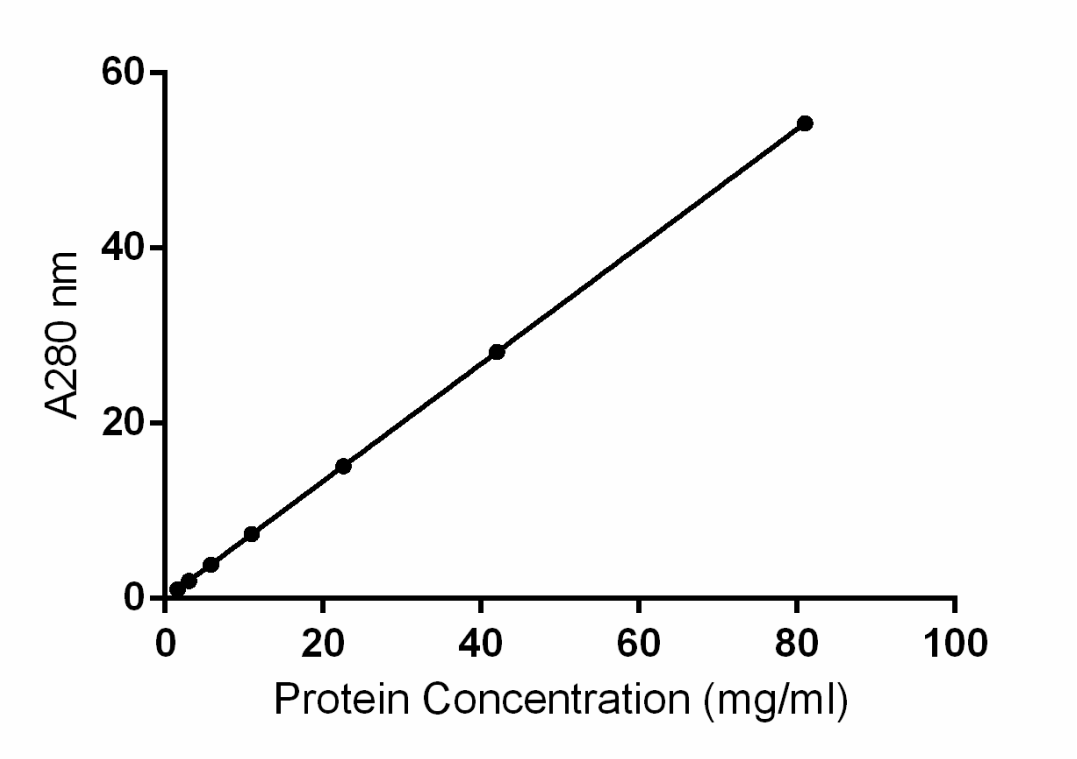


Figure 5.8: A standard curve of absorbance at 280 nm constructed using a series of known concentrations of BSA diluted in PBS. This curve was used to determine the protein concentrations of a panel of inactivated PT preparations by extrapolation of their absorbance to their concentration. The absorbance of each solution was measured in triplicate and standard deviation error bars generated.

Table 5.2: Estimated protein concentrations for each PT preparation. The protein content of each inoculum that was used to inoculate rabbits in order to generate specific sera was determined by absorbance at 280nm with reference to a standard curve of absorbance (Figure 5.8).

Inoculum	Protein Concentration (mg/ml)
CVS	5.11
CVS to LBV I	4.88
LBV to CVS I	4.75
LBV to CVS IIaIIb	4.77
LBVFSS	5.06
LBV A	4.99
LBV B	5.01
LBV C	4.99
WCBV	3.66
IKOV	3.71

between the serum titres determined by the two methods. The tires of each of the ten serum samples as determined by both PNA and FAVN are shown in Table 5.3. A paired Student's t-test was performed on the titres determined by both PNA and FAVN in order to determine whether there was any significant difference between the values produced by each assay $t(3) = 1.54$, $p = 0.05$. There was no significant difference between the pairs which confirms that the PNA method of determining serum titre is as accurate as the gold standard FAVN test and lends support for the titres of the sera generated against divergent lyssavirus PTs as these could only be calculated by PNA due to a lack of availability isolates for each of the live divergent viruses. Additionally no correlation was found between the protein concentration of inocula and the resulting anti-serum titre; $R^2 = 0.09$, $p = 0.311$.

Once accurate serum titres of each of the rabbit derived sera had been determined an investigation into the neutralisation profiles of the antigenic site swap sera was conducted. The CVS to LBV I, LBV to CVS I, LBV to CVS IIaIIb and LBVFSS specific sera were run against wildtype CVS and LBV PTs at 0.5 IU/ml (Figure 5.9). The CVS to LBV I serum neutralised the CVS PT but also neutralised the LBV PT by 47%. In a reciprocal experiment the LBV to CVS I serum completely neutralised the LBV PT but the CVS PT was only neutralised by 7% which was to a lesser degree than the reciprocal serum was able to neutralise LBV. Interestingly the LBV to CVS IIaIIb serum neutralised the CVS PT more strongly than the LBV PT; by 93% and 64% respectively. Finally, the LBVFSS specific serum neutralised both CVS and LBV PTs to a similar extent; 51% and 56% respectively. All of these neutralisation profiles were significantly different to neutralisation by negative dog serum except neutralisation of CVS PT by LBV to CVS I serum.

Table 5.3: Titres of polyclonal rabbit sera against CVS by PNA and FAVN. (A) Serum titres of rabbit derived polyclonal serum against inactivated PT preparations determined by standard PNA. (B) Serum titres of rabbit derived polyclonal serum against inactivated PT preparations determined by standard FAVN.

(A) PNA

Rabbit Sera	Titre (IU/ml)	Reciprocal Titre
CVS	512.0	40959.9
CVS to LBV I	4.0	320.0
LBV to CVS I	0.50	40.0
LBV to CVS IIaIIb	1.0	80.0
LBVFSS	4.0	320.0
LBV A	No neutralisation	No neutralisation
LBV B	No neutralisation	No neutralisation
LBV C	No neutralisation	No neutralisation
WCBV	No neutralisation	No neutralisation
IKOV	No neutralisation	No neutralisation

(B) FAVN

Rabbit Sera	Titre (IU/ml)	Reciprocal Titre
CVS	631.2	25904.27
CVS to LBV I	276.9	11363.98
LBV to CVS I	0.13	5.20
LBV to CVS IIaIIb	0.87	35.53
LBVFSS	7.79	319.81
LBV A	0.13	5.2
LBV B	0.13	5.2
LBV C	0.17	6.8
WCBV	0.13	5.2
IKOV	0.13	5.2

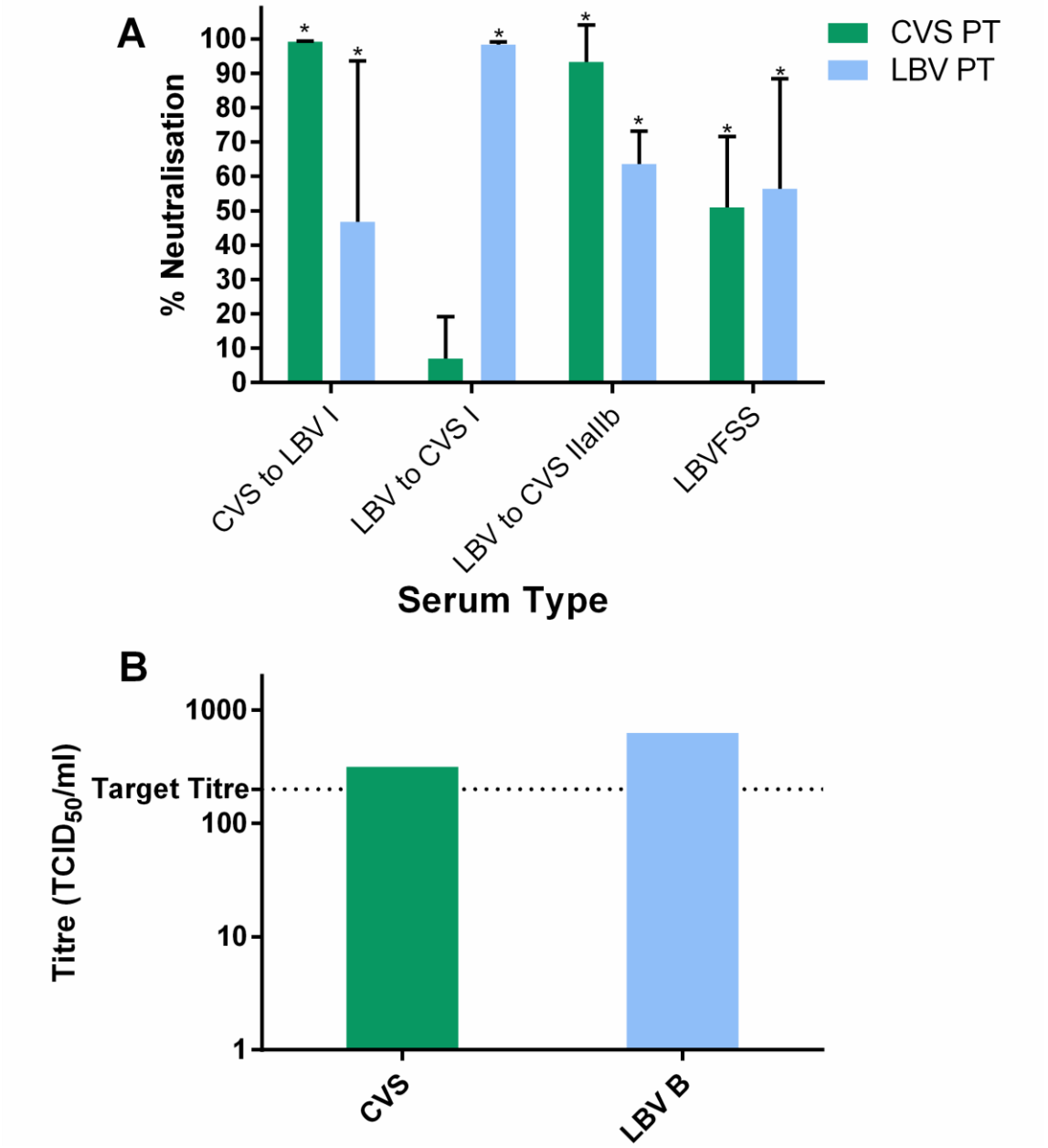


Figure 5.9: Rabbit derived antigenic site mutant sera PNA. (A) The four sera directed against antigenic site swap mutants were diluted to 0.5 IU/ml and run against wildtype CVS and LBV PTs. A 2-way ANOVA was used to determine any significant differences between neutralisation of each PT by the test sera compared to the lack of neutralisation by negative dog serum (0% neutralisation by negative dog serum data not shown). Significant differences are indicated by asterisks; *p≤0.05 (B) Back titrations of each PT used in the assay. The dotted line indicates the target titre following PT dilution. Error bars represent standard deviation (SD) about the mean of three replicate in-assay neutralisation tests.

5.5 Discussion

The PNA system was successfully optimised and used to conduct a variety of neutralisation experiments exploring the relative importance of each of the antigenic sites in a phylogroup specific neutralisation response as well as investigating the degree of cross neutralisation across different phylogroups.

From the site swap assays three antigenic sites were highlighted as being potentially important for both a phylogroup I and II neutralisation response. The CVS to LBV assay suggested those sites involved in phylogroup II neutralisation. Antigenic site II mutants were most strongly neutralised by both RABV and LBV specific sera indicating their essential role in neutralisation. Antigenic site I and III mutants also showed an alteration in their neutralisation profile by LBV specific sera however these mutants were less strongly neutralised than the site II mutants indicating these may play a more minor role.

A different neutralisation profile was evident for the reciprocal LBV to CVS site swaps as the data suggested that sites I and III were of potential importance however there was a lack of significance in these neutralisation profiles which must be considered when interpreting the patterns of neutralisation. The LBV wildtype PT was shown to potentially express a reduced amount of G on its surface than the CVS PT which meant that a reduction in the concentration of serum exposed to the LBV PT panel resulted in a clearer neutralisation profile. The reason for a reduced concentration of G on the LBV PTs is unknown however this may result from a lack of control of expression of G on the transfected cell surface or the absence of a stabilising interaction with the matrix protein within the PT particle. It may also simply be a reflection of the transfection efficiency or variation in the number of cells in the wells. Despite this, the results of both the 0.5

IU/ml and the 0.05 IU/ml PNAs suggest that a phylogroup I specific neutralising response may depend more heavily on antigenic sites I and III whereas a phylogroup II response is more dependent on antigenic site II. There is little evidence in the literature defining the importance of individual antigenic sites in lyssavirus neutralisation however what data exists is focussed on RABV viruses of phylogroup I in mice. This data indicates that sites II and III are the most important for a CVS neutralising response in *H-2*^{d} mice (Benmansour *et al.*, 1991). The importance of these two sites as well as site I is also supported by the finding that a large number of neutralising anti-glycoprotein mAbs are specifically directed towards these sites (Lafon *et al.*, 1983). The data collected in this investigation highlighting the importance of sites I and III in a phylogroup I neutralising response is therefore supported by the currently available evidence.

Now that the three sites which appear to play the most important role in phylogroup I and II neutralisation have been proposed this can direct the selection of potentially cross neutralising candidates. The full antigenic site swaps are an obvious choice as these contain all of the main immunodominant epitopes of each glycoprotein however, from this data it is possible that a mutant containing antigenic sites I and III of CVS and site II of LBV would be the most promising cross protective candidate. Unfortunately due to time constraints the generation of this mutant was not possible. However the generation of such a mutant would be a very interesting direction in which to take any future work.

The inclusion of two different RABV specific sera in this investigation; WHO and VLA was interesting as these are sourced from two different species; WHO from humans and VLA from dogs. The WHO serum is generated from a pool of human vaccinees.

However, production of this serum is problematic and relies on obtaining sera from individuals that may have been vaccinated with different inactivated preparations of rabies virus. This factor may have an impact on the neutralisation profile of this serum as there are numerous variations between different RABV strains prepared for vaccine generation although they are at least conserved across the major antigenic epitopes investigated in this study. The WHO serum is obtained from the WHO bank as an international reference serum. In contrast, the VLA serum is generated from a pool of vaccinated dogs for use within the APHA diagnostic laboratory. VLA serum is not available as an international standard however the concentration of rabies specific antibodies in each ampoule is carefully controlled and measured using the prescribed OIE standard techniques for assessment of neutralising antibodies in sera. As with the WHO serum, the exact vaccination history of each dog included in the VLA pool is unknown. Despite this, these sera represent key reagents for diagnostic evaluation and as such their utilisation in this study is well founded.

Both sera are directed against classic RABV isolates and as such their neutralisation profiles were expected to be similar. This was the case with most of the results however for a limited number of antigenic site mutant PTs there was a distinct difference between the degrees of neutralisation by the two RABV specific sera. The LBV to CVS IIa PT was more strongly neutralised by the WHO human serum than the VLA dog serum whereas the LBV to CVS IV PT was more strongly neutralised by the VLA serum than by the WHO human serum. This suggests there may be a difference between human and dog immune responses to the distinct antigenic sites and these differences may stem from a difference in the major histocompatibility complex (MHC) genes between species. Both humans and dogs have three classes of MHC genes however dogs have

two additional class I genes compared to humans and these are found on chromosomes 7 and 18 (Yukhi *et al.*, 2007). This difference in number of class I MHC genes may have impacted the humoral response as studies in dendritic cells (DC) show that RABV is presented by class I MHCs in mice (Irwin *et al.*, 1999). Though there is no direct evidence to demonstrate RABV presentation by MHC class I in dogs or humans it can be supposed that RABV would be presented by the endogenous pathway due to the absence of virus in blood and other extracellular compartments. Therefore the differences between canine and human humoral responses may be responsible for the variations seen in the neutralisation profiles of the sera derived from these differing species.

Overall a clear combination of antigenic sites playing an essential role in phylogroup I and II neutralisation has been identified, despite the variation in the results clear patterns were evident which led to the identification of antigenic sites I, II and III as those most important in lyssavirus neutralisation.

A further area of interest was the selection of a panel of PTs to generate a panel of polyclonal sera from rabbits. The PT panel was selected based on the availability of existing sera as well as the inclusion of a selection of antigenic site swap PTs which had been identified as promising cross protective candidates. Regulations surrounding the inoculation of genetically modified material into rabbits dictated that each PT was inactivated prior to inoculation into rabbits and as such an adjuvant was included to increase the local immune response and subsequent seroconversion. All rabbits seroconverted satisfactorily enabling the provision of sera specific for each of the novel PTs. A PNA assessing each serum sample against its homologous PT demonstrated that

the rabbits inoculated with CVS to LBV I, LBVFSS and LBV A had raised a particularly strong neutralising antibody response. Other titres were lower although the basis for these differences remains undefined although it is possible that individual differences in immune response between rabbits generated the diversity seen. Despite the strain of New Zealand White rabbits being an inbred strain there have been instances of immune variation in inbred laboratory models (Sellers *et al.*, 2012).

It has previously been shown that PT viruses are able to cause strong seroconversion in mice without prior inactivation, sufficient to induce protection against wildtype challenge virus (Powell *et al.*, 2012; Tao *et al.*, 2013) so it is of interest that in this study seroconversion was obtained in rabbits inoculated with inactivated PT. The sera obtained from these rabbits were then used to predict protection using the *in vitro* PNA.

An initial experiment, primarily to validate the method of antibody quantification by PNA in comparison with FAVN determined the antibody titre of each serum against CVS PT or live virus. The results indicated that there was no significant difference between titres determined by PNA or FAVN which validated the use of the PNA for this purpose as well as highlighting some cross neutralisation of CVS by a number of different sera. As expected CVS serum most strongly neutralised the CVS wt PT and live CVS virus. However, CVS to LBV I and LBVFSS sera also strongly neutralised CVS. This is interesting as both contain the antigenic sites of CVS, except CVS to LBV I lacks CVS site I, which suggests that these mutations confer a considerable level of neutralisation by CVS specific serum which implies these sera would be able to neutralise many other phylogroup I viruses. This also confirmed that the LBVFSS construct is susceptible to neutralisation by phylogroup I specific sera so if it retains

susceptibility to phylogroup II sera this would be a very promising cross neutralising construct.

Further investigation into the neutralising capabilities of these rabbit derived site swap sera suggested that the antigenic site swaps had altered the neutralisation profiles with respect to sera generated against wildtype viruses. The results of the site swap sera versus wildtype CVS and LBV PTs acted as a reciprocal experiment of the site swap PTs versus RABV and LBV specific sera. Comparison of the results from the reciprocal assays shows a different pattern of neutralisation in each. The CVS to LBV I serum versus CVS and LBV PTs shows a very similar neutralisation profile to the reciprocal assay using the antigenic site swapped PT whereas the LBV to CVS results were less similar. The LBV to CVS PT panel suggested that sites I and III were most important but the LBV to CVS I serum did not neutralise CVS PT whereas the LBV to CVS IIaIIb serum did. This suggests that site II is more important than site I in a neutralising response. The LBVFSS serum was able to neutralise both CVS and LBV PTs by approximately 50% which is in contrast to the ability of both RABV and LBV specific sera to strongly neutralise LBVFSS PT. The differences between these neutralisation profiles may be due to innate differences between human, rabbit, dog and mouse immune responses as the antigenic sites were originally mapped using murine antibodies (Lafon *et al.*, 1983) whereas the sera used in this investigation originated from a range of different mammalian species which may respond to antigens in a different manner to each other. It would be valuable to run the site swap rabbit sera against LBV virus in a modified FAVN assay in order to determine whether the neutralisation profiles between LBV PT and LBV virus are statistically similar, as was shown with CVS PT and virus.

Chapter 6: Investigation of neutralisation by antigenic site directed mAbs

6.1 Introduction

A further application of the PNA using antigenic site swap PTs is their utility in the potential characterisation of monoclonal antibody epitopes. The lyssavirus glycoprotein is the main immunogenic component of the virus as it is the only viral protein expressed on the virion surface. This means that many neutralising antibodies are directed towards this protein and many specifically towards the antigenic sites as these are the proposed immunodominant regions (Section 1.1.2.4.1).

Monoclonal antibodies have recently been proposed as an alternative to rabies immunoglobulins (RIG) (Section 1.2.2) (Both, 2013; Muller *et al.*, 2009) for the passive immunisation of humans following the suspicion of a bite from a rabid animal. RIG is composed of polyclonal antibodies from either horses or humans. The current procedure for PEP involves administration of RIG in and around the wound site utilising a dose:weight ratio of 20 IU/kg for HRIG and 40 IU/kg for ERIG. As a result for each potential exposure a large amount of RIG may be required. Importantly, it is estimated that between 10-16 million people receive PEP annually (Bourhy *et al.*, 2010; Warrell, 2010). This poses significant disadvantages in that human serum requires multiple downstream processes in order to reduce the risk of transmission of blood-borne infectious disease. In addition, the production of ERIG has been discontinued by many international manufacturers due to ethical issues raised by animal protection groups. This means that supply relies on regional manufacturers e.g. in Thailand. The most significant disadvantage, however is the very high cost of RIG; HRIG at $100-250 per

dose and ERIG at $25-50 per dose (Knobel *et al.*, 2005; Muller *et al.*, 2009). These high costs preclude the use of RIG in areas of Africa and Asia where it is most needed.

Due to these constraints with RIG, the potential application of monoclonal antibodies (mAbs) as an alternative is very attractive. This approach would utilise antibodies that are directed towards the lyssavirus glycoprotein, often targeting specific antigenic sites along the glycoprotein. For many mAb preparations, the epitope binding regions have not yet been determined and so the potential for antigenic variation and the significance of variation on the ability of mAbs to neutralise virus has not been assessed. However, due to the immunodominance of the antigenic sites within G, they are often the target of potently neutralising mAbs. To test this, and evaluate mAb binding sites for some commonly used mAbs the panel of PTs containing antigenic site swaps were used to assess the ability of different mAbs to neutralise them.

The panel of mAbs investigated in this study is shown in Table 6.1. mAbs E559 and 62-7-13 have already been acknowledged by the WHO as potential mAbs for use in PEP (Muller *et al.*, 2009) whilst mAb D1 is currently only used in a serological diagnostic capacity in a commercially available enzyme linked immunosorbent assay (ELISA) (Fournier-Caruana *et al.*, 2003). mAb D8 was produced at APHA and has been shown by fluorescent antibody virus neutralisation test (FAVN) to specifically neutralise DUVV so for this reason has been excluded from inclusion in potential therapeutic cocktails. However the epitope that D8 binds to on the glycoprotein has not yet been defined and so it was included in the study.

6.2 Utilisation of a panel of PTs to investigate binding sites for monoclonal antibodies

The panel of antigenic site swapped PTs between phylogroups I and II generated in Section 3 was used to investigate the potential binding regions of a panel of mAbs, some of which have defined antigenic sites within the proposed epitopes of the lyssavirus glycoprotein. As none of the antibodies to be investigated were able to neutralise phylogroup II lyssaviruses by FAVN (David Selden- personal communication and (Muller *et al.*, 2009)) the glycoproteins with antigenic site swaps between phylogroups I and II were used to assess the specificity of the different monoclonal antibodies.

Each of the monoclonal antibodies had previously been proposed to target different antigenic epitopes on the glycoprotein. Earlier studies had suggested that E559 targeted antigenic site II (Muller *et al.*, 2009) so all PTs containing mutations in antigenic site II were tested against this mAb. Likewise, cross neutralisation studies have suggested the 62-7-13 mAb targets antigenic site I so this mAb was tested against all PTs with antigenic site I swaps. Finally, D1 was tested against all PTs with antigenic site III swaps. Details of each mAb used in this investigation are given in Table 6.1. The results of the PNAs are shown in Figure 6.1. The titre of each of the mAbs was determined via FAVN and the dilution required to give 0.5 IU/ml was calculated. The value of 0.5 IU/ml was selected as this is the OIE and WHO approved threshold antibody titre that confers protection against infection with RABV. It is therefore used as a standard in all OIE and WHO gold standard serological tests. Each dilution of 0.5 IU/ml was then carried out and the titres confirmed on a subsequent FAVN. The PTs were then exposed to mAbs at these dilutions and the degree of neutralisation of each PT was calculated

Table 6.1: Technical information for the panel of mAbs utilised in this investigation. ERA – Evelyn Rokitniki Abelseth SAD derived RABV strain, PV – Pasteur virus, a RABV strain, IgG – immunoglobulin isotype G. Adapted from (Muller *et al.*, 2009).

	E559	62-7-13	D1	D8
Proposed Antigenic site recognised	II	I	III	Unknown
Method for determining epitope	Sequencing (following generation of an escape mutant)	Cross neutralisation	Unknown	Unknown
Antigen	ERA G	Whole ERA	Whole PV	Whole DUVV
Fusion partner	P3-X63Ag8	Sp2/0–Ag14 myeloma	Unknown	NS1 myeloma
IgG subtype	IgG 1	IgG 2b	IgG 1	Unknown

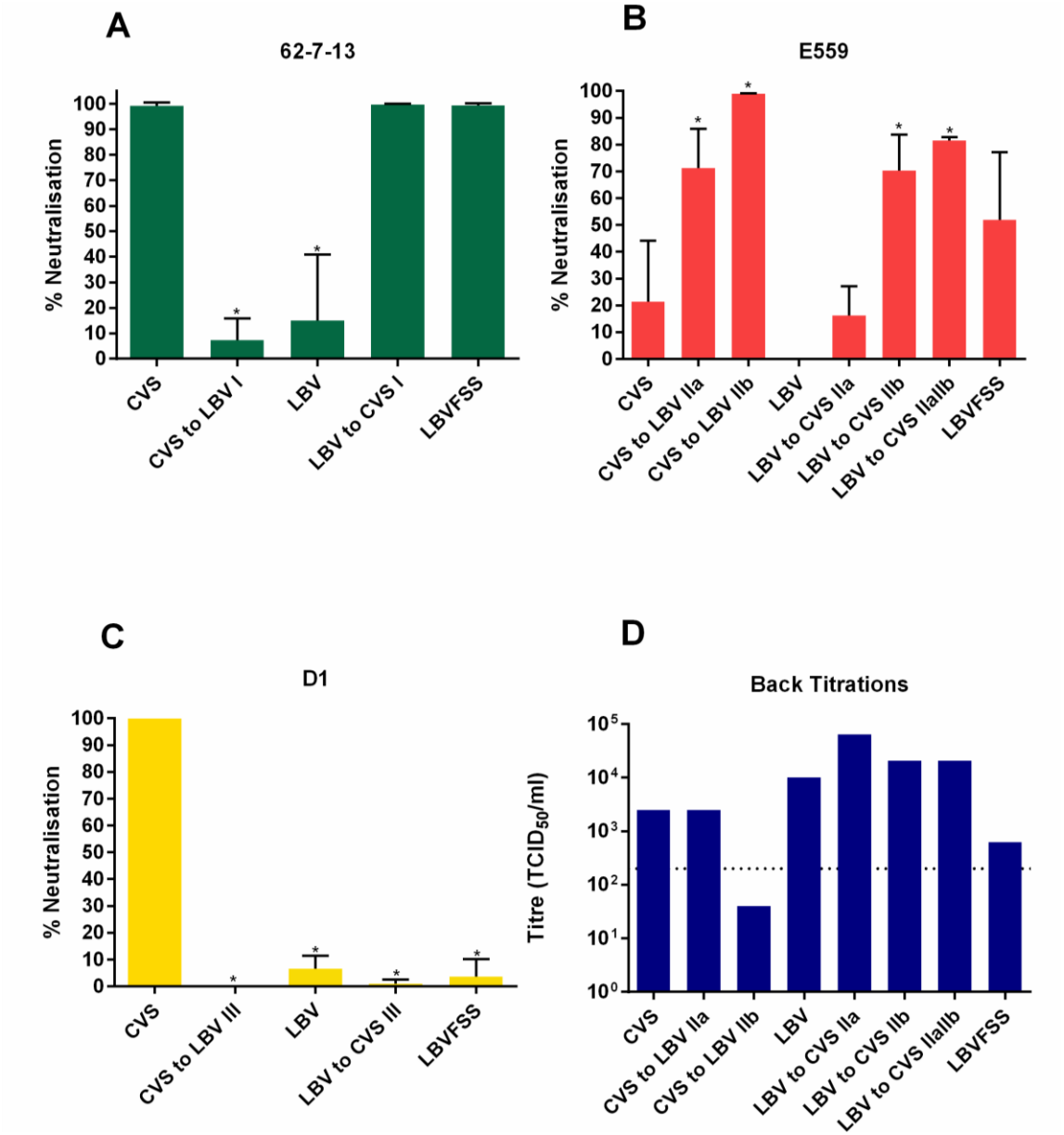


Figure 6.1: Neutralisation of antigenic site swap PTs by a panel of mAbs at 0.5IU/ml. (A) neutralisation profile of antigenic site I swap PTs against mAb 62-7-13. (B) Neutralisation profile of antigenic site II swap PTs against mAb E559. (C) Neutralisation profile of antigenic site III swap PTs against D1. (D) Back titrations of each PT used in the assay. The dotted line indicates the target titre following PT dilution. Error bars represent standard deviation (SD) about the mean of three replicate in-assay neutralisation tests. A one way ANOVA was used to determine significant differences between neutralisation of each PT by each mAb and neutralisation of CVS by each mAb. Significance is indicated by an asterisk; *$p \leq 0.05$.

as a function of the reduction in luminescence in the presence of mAb as opposed to luminescence in the absence of mAb.

Antigenic site I is the proposed epitope of 62-7-13 (Both *et al.*, 2013) and this has been confirmed by its neutralisation profile against antigenic site I PTs (Figure 6.1 (A)). CVS, a phylogroup I lyssavirus was 99% neutralised by 62-7-13, as were LBV to CVS site I and LBV to CVS full antigenic site swaps. All of these PTs contain the phylogroup I antigenic sites, with this being the only phylogroup I sequence in the LBV to CVS I construct. In contrast the wildtype LBV PT as well as the CVS to LBV site I PT were only very weakly neutralised, by 15% and 7% respectively. Both of these constructs contain a phylogroup II antigenic site I sequence which was clearly not neutralised by 62-7-13. Due to the CVS to LBV site I PT being so poorly neutralised, this indicated that 62-7-13 targets specifically and solely the phylogroup I antigenic site I as no other PTs underwent any considerable level of neutralisation. The ability to use phylogroup II G containing PTs with just the phylogroup I antigenic sites enabled confirmation of the data proposed previously by (Both *et al.*, 2013).

In contrast to the clearly specific 62-7-13 CVS site I epitope, the results of the assessment of E559 binding and neutralisation were more complex (Figure 6.1(B)). The wildtype CVS PT was neutralised by only 21%, despite having the same sequence in antigenic site I as the ERA strain (Table 6.2) against which E559 was generated. The LBV to CVS site IIaIIb PT was strongly neutralised by 82%, however, which does suggest that the phylogroup I antigenic site II plays a role in E559 neutralisation. This was confirmed by the LBV to CVS full antigenic site swap PT being 52% neutralised however LBV to CVS IIa was only 16% neutralised which suggests that antigenic site

Table 6.2: Alignment of CVS, ERA and LBV lineage B antigenic sites. Residues in red differ from the CVS sequence. This highlights the close sequence similarity between the RABV strains; CVS and ERA and the divergence of phylogroup II LBV sequence.

Virus	Site IIb	Site IIa	Site I	Site IV	Site III
CVS	GCTNLSEFS	KRA	KLCGVL	FH	KSVRTWNEI
ERA	GCTNLSGFS	KRA	KLCGVL	FR	KSVRTWNEI
LBV	GCGTSSVFS	KKS	TLCGKP	NR	LKVDNWSEI

IIb plays a stronger role in E559 neutralisation than IIa. This role of site IIb was confirmed by the reciprocal PT swaps as the CVS to LBV IIa PT, which still contains CVS site IIb, was 71% neutralised. The CVS to LBV IIb PT however appeared to be more strongly neutralised than the IIa swap which reduces the certainty of the role of CVS IIb in E559 neutralisation.

The neutralisation profile of mAb D1 against all of the antigenic site III swap PTs suggests that site III may not be a target of D1 (Figure 6.1 (C)). Only the CVS wildtype PT was completely neutralised by D1. All of the remaining site III PTs were not neutralised at all or to low levels. The LBV to CVS III and LBVFSS PTs both contain the CVS antigenic site III however these were only very weakly neutralised, by 1% and 4% respectively. These degrees of neutralisation are similar to those of wildtype LBV and CVS to LBV III which both contain the antigenic site III of LBV which suggests site III may be necessary for D1 neutralisation but is not sufficient; other sequences are required.

6.3 Escape from neutralisation by the proposed mAb panel

The cocktail of mAbs being considered by the WHO as a potential alternative to current RIG therapy, E559 and 62-7-13 have been shown in general to be able to neutralise RABV viruses (Section 6.2 and (Muller *et al.*, 2009)) however there have been instances where certain phylogroup I viruses have been demonstrated to escape neutralisation by these mAbs (Muller *et al.*, 2009). Data from Section 6.2 suggested that mutations to CVS antigenic site I inhibited neutralisation by mAb 62-7-13 and mutations to site II influence E559 neutralisation, thus the sequences of antigenic sites I and II in a number of viruses known to escape neutralisation were examined in order to identify any

specific residues which may determine neutralisation. The viruses and their antigenic site sequences can be seen in Table 6.3.

A critical role for residue Lys 245 in 62-7-13 neutralisation was identified (Both *et al.*, 2013) and it was found that an escape mutant of E559 had mutations of Leu to Arg at 57 and Lys to Glu at 217 (Muller *et al.*, 2009). All three of these residues are located in antigenic sites: 245 in site I and 57 and 217 in site II. It was therefore postulated that any mutations at these positions would affect neutralisation by the respective mAbs. It can be seen from Table 6.3 that both PV and EBLV-1 were not neutralised by 62-7-13 and it was clear that both contain mutations at position 245 with Glu and Arg residues in place of Lys, respectively. This confirms the critical role of Lys 245 as well as explaining the lack of neutralisation of PV, EBLV-1 and all PTs containing LBV antigenic I.

Kelev is a strain of RABV and has been repeatedly shown to escape neutralisation by E559 (Muller *et al.*, 2009). From Table 6.3 it was clear that Kelev contains mutations in site II at the same positions as the E559 escape mutant. This suggests that these residues may have a critical role in E559 neutralisation, however the USA Bobcat isolate had the same sequence in site II as both CVS and PV which were neutralised by E559. This suggested that there may be epitopes outside of antigenic site II which play a role in E559 neutralisation and it may be that these mutations affect the conformation of site II as it is the only discontinuous epitope on the lyssavirus glycoprotein so is likely to be the most susceptible to be affected by mutations resulting in conformational changes. Despite the Bobcat isolate neutralisation profile against E559, the significance of the residues at positions 57 and 217 in the Kelev RABV strain was further investigated. An isolate of Kelev was available in the laboratory so a series of PCR products were

Table 6.3 : Sequence of antigenic sites and neutralisation profiles of a panel of viruses which escape neutralisation by the current mAb cocktail being considered by WHO as an alternative to current serological therapies. Residues in red differ from CVS-11 which is neutralised by both mAbs in the cocktail.

Virus	Site I (245-250)	62-7-13 Neutralised	Site IIa (217-291)	Site IIb (53-61)	E559 Neutralised
CVS-11	KLCGVL	Y	KRA	GCTNLSGFS	Y
PV	QLCGVL	N	KRA	GCTNLSGFS	Y
Kelev, Israel	KLCGVL	Y	TRA	GCTNRSEFS	N
Bobcat, USA	KLCGVL	Y	KRA	GCTNLSGFS	N
EBLV-1, Germany	RLCGVL	N	KKA	GCTTLTPFS	Y

generated from different sources of Kelev: from infected mouse brain homogenate and from an infected tissue culture cell pellet. Previously next generation sequencing (NGS) using the 454 platform had identified four different variants of Kelev, each with a different combination of aa at positions 57 and 59 (Denise Marston – unpublished data). The variation at these sites was of interest due to their presence in antigenic site IIb so in order to determine whether the same variants were present in the mouse brain homogenate or the cell pellet PCR reactions were set up to generate the entire G ORF as product. These 1.5 kb PCR products were sequenced using the MiSeq platform (Illumina) in house at the APHA Central Sequencing Facility and the results were analysed using Tablet (version 1.14.10.20). The consensus sequence used to map the reads was the glycoprotein ORF of rabies virus isolate RV2324 (KF154998.1).

A number of nucleotide (nt) variants were identified in the cell pellet product whereas only one nt variant was found in the mouse brain PCR products. The variant identified in all PCR products was a T to G at position 170. This nt variant was identified in 99.9% of all mouse brain reads and 72.4% of all cell pellet reads suggesting that this variation is under substantial selective pressure. In addition to the nt 170 variant in the cell pellet, a variant consisting of a C to T substitution at position 169 was identified in 32.2% of all reads. There were also a limited number of variants with a G to A substitution at position 175 and some with an A to G substitution at position 176. All of these variations are within antigenic site IIb and result in amino acid changes in the glycoprotein sequence.

The significance of the substitutions at positions 169 and 170 is that these determine which aa will be present at position 57 in the glycoprotein; the precise residue which appears to influence E559 neutralisation. The C to T substitution at 169 in combination

with the T to G substitution at 170 results in a tryptophan residue at aa 57 whereas the 170 substitution alone results in an arginine residue at aa 57. The consensus sequence of Kelev along with CVS, PV and EBLV-1 contain a leucine residue at position 57 whereas those viruses which escape neutralisation by E559; the Kelev isolate from this investigation along with MOKV and IKOV (David Selden – unpublished data) contain arginine, glutamate and glycine residues at position 57, respectively. This therefore led to the hypothesis that leucine 57 plays a critical role with regard to mAb E559 neutralisation.

6.4 Neutralisation profile of mAb D8

The epitope of mAb D8 has not yet been defined however preliminary virus neutralisation assays suggest that D8 is only able to neutralise DUVV, the virus against which it was generated. In order to investigate further the neutralisation profile of this mAb it was run against a panel of PTs representing every lyssavirus to determine whether it is able to cross neutralise any other PTs. The result of this PNA can be seen in Figure 6.2.

mAb D8 showed the expected neutralisation profile against DUVV PT. Despite DUVV being a phylogroup I lyssavirus this anti-DUVV mAb shows a very limited neutralisation capacity against any other phylogroup I PT, unlike the RABV specific sera in Chapter 4. The only phylogroup I PT showing significant neutralisation by D8 is BBLV which was 88.5% neutralised. The MOKV PT was 72% neutralised and this was the only other PT that showed more than 50% neutralisation. Interestingly both LBV B and IKOV showed no significant difference in neutralisation when compared to DUVV using a one way ANOVA as both were 45% neutralised.

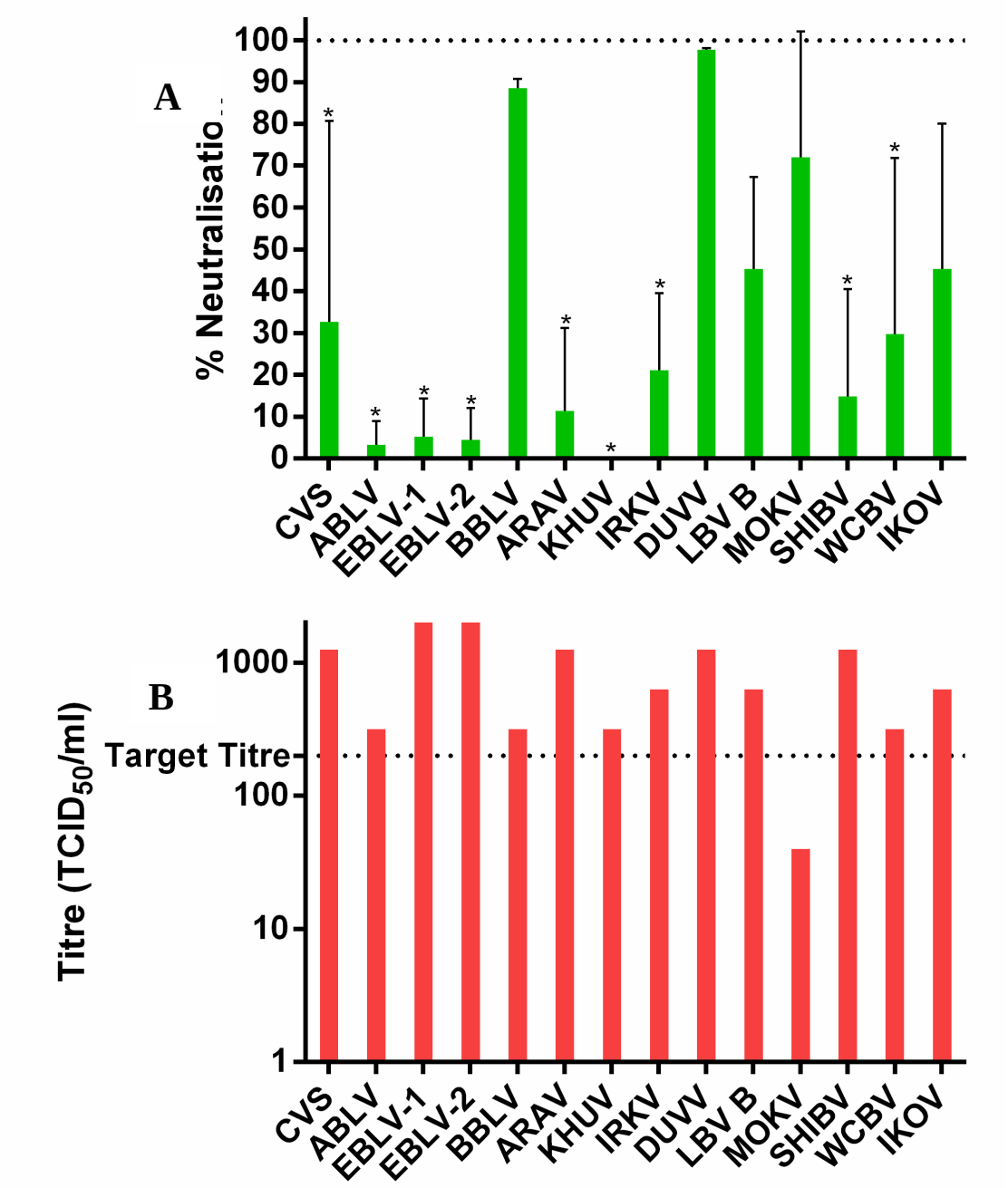


Figure 6.2: Neutralisation profile of mAb D8 against a full panel of wildtype lyssavirus PTs. (A) PT neutralisation assay. mAb D8 was diluted to 0.5 IU/ml and a one way ANOVA compared neutralisation of each PT to neutralisation of DUVV. Significant differences are indicated by an asterisk. Error bars represent standard deviation (SD) about the mean of three replicate in-assay neutralisation tests. A one way ANOVA was used to determine significant differences between neutralisation of each PT by D8 and neutralisation of DUVV by D8. Significance is indicated by an asterisk; *$p \leq 0.05$. (B) Back titrations of each PT used in the assay. The dotted line indicates the target titre following PT dilution.

Due to the insignificant neutralisation of the CVS PT by mAb D8 and the significant yet limited neutralisation of LBV B this mAb was not run against the antigenic site swap panel of PTs as in Figure 6.1.

6.5 Discussion

Knowledge surrounding the ability of monoclonal antibodies to bind epitopes within the glycoprotein is of great significance when considering the potential for antibody mediated escape and the role of mAbs as a replacement for RIG. Such information is of particular importance where the evaluation of neutralising antibodies is concerned as the potential for escapes mutant generation has significant implications on the potential utility of mAbs in a cocktail for the replacement of RIG. The neutralisation profiles of the mAbs investigated here do not entirely match with published data on proposed epitopes. There may be a number of reasons for this, however, the lack of a crystal structure for the rabies, or any lyssavirus glycoprotein, significantly hinders interpretation of whether the structure of Gs in mutated PT particles has been altered potentially affecting binding and neutralisation. A number of estimations as to the effect of individual amino acids (aa) within the protein may be generated by looking at the physiochemical properties of the amino acids both within the CVS and LBV antigenic sites as well as residues adjacent to these sites. Any changes in residues within sites may alter the physiochemical interactions between residues within the protein, thus affecting the structure and subsequently recognition by site specific mAbs. The aa sequence of the antigenic sites in both wildtype and the antigenic site swap mutants along with the physiochemical properties of the adjacent residues are shown in Figure 6.3.

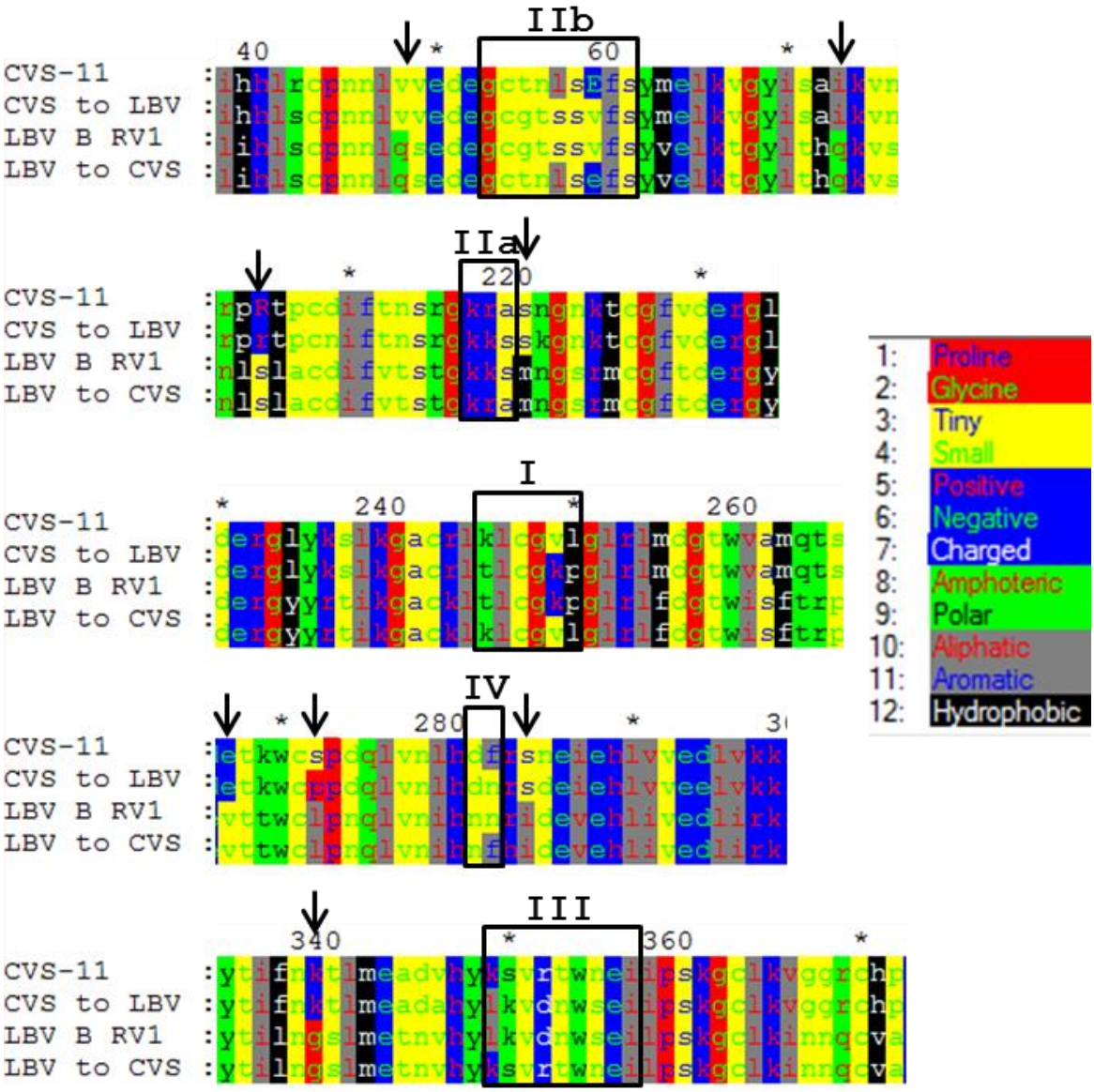


Figure 6.3: Alignment of CVS-11 and LBV RV1 wildtype glycoprotein sequences along with reciprocal antigenic site swap sequences made in GENEDOC. Residues are shaded according to their physiochemical properties. Antigenic sites are marked in black boxes and positions at which a physiochemical swap occurs have been highlighted by arrows.

The GENEDOC alignment software assigned physiochemical properties according to those proposed by (Dickerson & Geis, 1969) and the amino acid properties assigned by (Betts & Russell, 2003) were also used to interpret the effects of aa changes. Antigenic site IIb contains two residues at which a switch in physiochemical properties of the amino acids is altered by the antigenic site switch. The aliphatic leucine (L) in CVS is switched to a tiny serine (S) and the negatively charged glutamate (E) is switched to a small valine (V) in the antigenic site swap. It has already been established however that these switches are only weakly unfavourable in terms of physiochemistry (see Table 3.2) but the folding of this antigenic site may be affected by the alteration in the adjacent amino acids. The residues upstream of the antigenic site retain very similar physiochemical properties between the CVS and LBV backbones however downstream; there is a switch from a hydrophobic methionine (M) in the CVS backbone to a small valine (V) in the LBV backbone. This may result in a conformational change due to the size difference between the two amino acids however both are hydrophobic so similar interactions should be maintained. Upstream of antigenic site IIa there is a switch from a positive arginine (R) in CVS to a small threonine (T) in LBV. Though threonine is marginally polar, the main difference between these two residues is that threonine is C-beta branched so contains two, rather than one, non-hydrogen substituent on the C-beta carbon. This results in a larger than average amino acid residue and indeed threonine is very rarely found in alpha helices but commonly found in beta-sheets. In contrast, due to the polar nature of arginine this residue commonly forms salt bridges with negatively charged residues to create hydrogen bonds which confer stability to the protein. Downstream of antigenic site IIa there are more physiochemical changes as the region becomes more hydrophobic in the LBV backbone. The hydrophobic methionine residues in LBV at this site would reside on the interior of the protein whereas the serine and

threonine residues in the corresponding positions in CVS are not hydrophobic. Serine is a small slightly polar residue which can be found on the interior of proteins, like methionine however as mentioned, threonine is a larger than average residue due its C-beta branch and is unlikely to reside in alpha helices. This gives some insight into the potential structures which have been altered in the site swap mutants. For example this may help explain the unexpected neutralisation profile of E559 as this was proposed to recognise antigenic site II however it did not seem to show a profile consistent with this. If the alteration in the backbone had indeed affected the structure of this discontinuous epitope then the CVS to LBV PTs would be expected to have been more strongly neutralised than the LBV to CVS PTs. This pattern does seem to be present with both the CVS to LBV site IIa and IIb PTs being very strongly neutralised. In confirmation, both the LBV to CVS IIa and IIb PTs were less strongly neutralised than their reciprocal counterparts which suggests that the alteration in the residues surrounding these antigenic sites did have a noticeable effect on the ability of E559 to recognise and neutralise these mutants. This may also suggest that it is the combination of antigenic sites IIa and IIb of CVS that is essential for E559 neutralisation as the LBV to CVS IIaIIb PT was 82% neutralised which suggests that the sequence of the combination of antigenic sites IIa and IIb remains of strong importance for E559 neutralisation, even if the surrounding residues and hence the structure of the discontinuous site may differ. Interestingly, E559 is capable of binding to phylogroup II lyssavirus G proteins as evidenced by its ability to detect LBV and MOKV G in immunofluorescence (Finke, S. personal communication), however it is unable to neutralise these lyssaviruses. This may indicate multiple binding residues on G of which some are important in neutralisation. The fact that the escape mutant generated by E559 contains two amino acid mutations; one in site IIb and the other in site IIa indicates that site II does play a role in binding

and neutralisation by this mAb. In addition to this, NGS data showed a consistent variant at nt 170 which encodes aa 57 within antigenic site IIb which further supports the role of site IIb in E559 neutralisation and highlights the need for further investigation of its epitope as this may have significance for the WHO's continued investigation of this mAb as part of a novel therapeutic cocktail.

The neutralisation profile of 62-7-13 was as expected with only PTs containing the CVS site I sequence being neutralised. The physiochemical features of the residues surrounding antigenic site I differ only very slightly between CVS and LBV which may explain the accuracy of the mAb. There is a switch of two hydrophobic methionines (M) in CVS to two aromatic phenylalanines (F) in LBV downstream of site I, however phenylalanine is strongly hydrophobic, as is methionine so these switches are unlikely to have affected the protein folding, thus site I was specifically targeted by 62-7-13.

The interest in mAb D1 stems from its use in the current rabies specific ELISA. It has been shown to recognise antigenic site III in native but not β-mercapto-ethanol and/or sodium dodecyl sulphate (SDS) treated G. It has been shown to have a strong neutralising potency towards RABVs: CVS, Pasteur virus (PV) and Pitman-Moore virus (PM) and also towards EBLV-2 in virus neutralisation assays (Fournier-Caruana *et al.*, 2003) so may have some potential for use in a therapeutic cocktail. However, D1 was only able to neutralise the wildtype CVS PT. The lack of neutralisation of PTs containing CVS antigenic site III by mAb D1 may be explained by the considerable alteration in the physiochemical properties of the residues downstream of site III between the CVS and LBV backbones. Upstream of site III there are very few changes

in physiochemical properties however nine residues downstream of site III there are a number of changes to the physiochemical features of the glycoprotein. The small valine (V) in CVS is switched for an aliphatic isoleucine (I), however, both of these residues, like threonine are C-beta branched so the impact of this switch is unlikely to cause any alteration in the protein structure. Following this likely insignificant switch there are a number of glycine (G) residues in CVS which are not present in LBV. This may have a significant impact on the folding of the protein as glycines contain a small hydrogen in their side chain, as opposed to carbons in all the other amino acids. This means that glycines confer a much greater amount of conformational flexibility than other amino acids as they can reside in areas of the protein which would be impossible for other amino acids. There may be a tight turn downstream of antigenic site III in CVS, due to the G residues which cannot be present downstream of the LBV site III and this may have had a severely detrimental effect on the structure/availability of antigenic site III. It has been proposed that antigenic site III forms a loop-like structure on the surface of the lyssavirus glycoprotein (Benmansour *et al.*, 1991) and this structure may be due to the presence of the glycines, enabling this tight turn. Thus, when the glycines are no longer present within the LBV to CVS site swap, the usual loop like conformation of site III is no longer possible resulting in mAb D1 being unable to recognise and bind to its usual epitope.

Overall it appears that the residues surrounding the antigenic sites play as an important a role in antigenic site formation as the sequences of the antigenic sites themselves. This has been demonstrated by the alteration in physiochemical properties of the glycoprotein both up and downstream of antigenic sites II and III between CVS and LBV resulting in

potentially altered presentation of these antigenic sites which may have had a negative impact on the ability of site specific mAbs to recognise or access their epitopes. The fact that the physiochemical properties of the glycoprotein around antigenic site I differ minimally between CVS and LBV may have enabled the expected neutralisation profile, which was seen in this experiment and this lends support to the importance of residues surrounding the antigenic sites in the formation and presentation of mAb epitopes. In addition the current limited data on the critical residues for 62-7-13 and E559 neutralisation has been supported by this investigation. The lack of neutralisation of any PT or virus containing a mutation at residue 245 by 62-713 was previously demonstrated (Both *et al.*, 2013). The residues 57 and 217 identified as potentially essential to E559 neutralisation (Muller *et al.*, 2009) were shown to be mutated in Kelev and were also mutated in the site II antigenic site swap mutants however the lack of neutralisation of the Bobcat isolate which contained wildtype residues at positions 57 and 217 and the lack of neutralisation of the CVS wildtype PT by E559 put the significance of these two residues into question. The NGS data however provides support for the critical role of leucine 57 in E559 neutralisation though further investigation into the epitopes of both E559 and D1 is required in order to confirm their specificities.

The neutralisation of the mAb D8 was also investigated against a panel of PTs representing all of the currently identified lyssaviruses. Initial virus neutralisation assays had shown D8 potently neutralised DUVV (unpublished data) however no investigation into its epitope had been conducted. The PNA (Figure 6.2) showed that D8 was able to significantly neutralise BBLV along with some of the other African lyssaviruses however it showed no potency against any phylogroup I PTs except BBLV which

suggests that the strong intra-phylogroup I neutralisation seen with vaccine induced polyclonal sera (Figures 4.1 and 4.2) may not be representative of all sera raised against each phylogroup I virus. Further investigation into the cross neutralising capacity of sera raised against all phylogroup I viruses against all other phylogroup I viruses/PTs would be valuable.

It was interesting that many of the African lyssavirus PTs were neutralised by D8 as a similar pattern was seen with IKOV specific polyclonal sera. This again may suggest that the African lyssaviruses share some antigenic features which likely reside outside of the defined antigenic sites.

Due to the limited neutralisation of CVS by D8 it was unlikely that any antigenic sites on CVS would comprise the epitope of D8, in addition LBV B was only 45% neutralised suggesting it did not contain any significant epitope for D8. This result means that the epitope for D8 likely lies outside of the currently defined antigenic sites however an assay including D8 against the CVS PNA panel may yield some information into the ability of D8 to neutralise LBV as the CVS PTs contain only the antigenic sites of LBV, thus if any were to be neutralised by D8 it would be highly likely that this neutralisation would have resulted from recognition of that particular LBV antigenic site as it has been shown that D8 is unable to neutralise CVS.

Chapter 7: Construction and characterisation of full length clones *in vitro* and *in vivo*

7.1 Introduction

In order to confirm the neutralisation results obtained using the PNA, a panel of recombinant full length clones were generated for subsequent neutralisation experiments with the aim of preparing these constructs for use in vaccination/challenge experiments *in vivo*. The data obtained from the PNA suggested that wildtype CVS and LBV clones alongside the full antigenic site swaps: CVSFSS and LBVFSS would be the most informative in the first instance. These recombinants were generated in the context of a vaccine strain RABV, cSN where the cSN glycoproteins were exchanged for the heterologous glycoproteins of interest. The functionality of successfully rescued clones was determined *in vitro* prior to inoculation of viable clones into vaccinated and unvaccinated mice to determine protection against challenge and pathogenicity of each virus.

7.2 Construction of full length clones

The full length cSN clone contains a number of restriction sites which enable manipulation of the genome (Figure 7.1). The G gene is flanked by *SmaI* and *NheI* restriction sites. Due to the presence of a *SmaI* site within the pCR Blunt vector (Invitrogen) used for subcloning, an alternative blunt ended restriction enzyme site was engineered upstream of the G genes, *HpaI*. This site was not present in either the subclone or full length clone. *HpaI* recognises a 6 nucleotide sequence; the same length as that of *SmaI* so does not result in any frame shifts and also generates a blunt end like *SmaI* so the blunt to sticky ended ligation was maintained. The *HpaI* and *NheI*

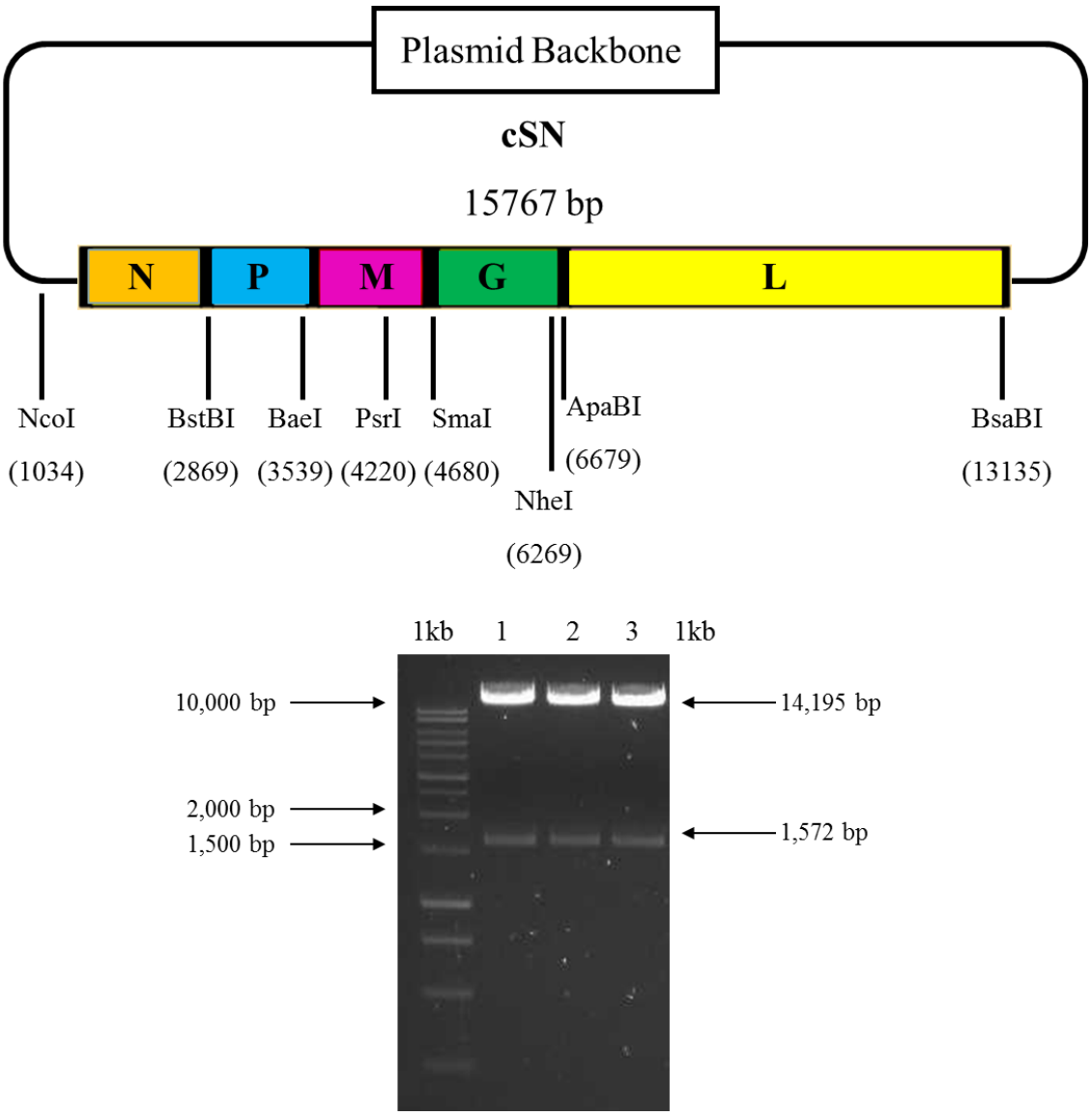


Figure 7.1: Graphic illustration of cSN, the full length cDNA copy of the RABV genome. All restriction enzyme recognition sites within the plasmid sequence which enable manipulation of the genome are highlighted. Mutated or wildtype glycoproteins of interest were introduced into the cSN backbone via *HpaI/PmeI* and *NheI* restriction enzymes. Digestion of the intact cSN plasmid with *SmaI* and *NheI* generated two fragments; the 14,195 bp backbone and the 1572 bp glycoprotein in lanes 1-3. The ladder is 1kb DNA ladder (Promega).

restriction enzyme sites were engineered via PCR to flank each of the mutated and wildtype lyssavirus G genes that were produced (see Section 3) (primer sequences in Appendix I).

The cloning strategy was the same for all produced G constructs (Figure 7.2). Firstly cSN was digested with *SmaI* and *NheI* to remove the G gene. The now linear cSN plasmid minus the G gene was then purified by LMP agarose gel electrophoresis. Meanwhile the insert constructs were directly digested by *HpaI* and *NheI*. After purification of the cSN vector, the pre-digested G constructs were then inserted into the vector by ligation.

This approach of directly ligating the PCR product into the vector was unsuccessful for all of the G constructs so an additional step of subcloning each of the inserts into a commercially available blunt ended vector was attempted. The PCR products resulting from the addition of the *HpaI* and *NheI* restriction enzyme sites flanking the G gene were ligated into the pCR Blunt vector. These ligations were then transformed into MAX Efficiency® DH5α-T1^{R} competent cells (Invitrogen) in the presence of the selective antibiotic, kanamycin. Putative positive clones were purified by the small scale plasmid preparation technique and confirmed by restriction enzyme mapping. Plasmid DNA from confirmed positive small scale DNA preparations was then produced in large amounts using the maximum scale plasmid preparation kit. The purity and concentration of the resulting large scale DNA preparations were measured on a spectrophotometer (Section 2.2.7). Following this, 10µg of each large scale plasmid preparation was digested with *HpaI* and *NheI* to excise the G insert from the blunt vector. Following digestion, the inserts were purified by LMP gel electrophoresis and the resulting insert

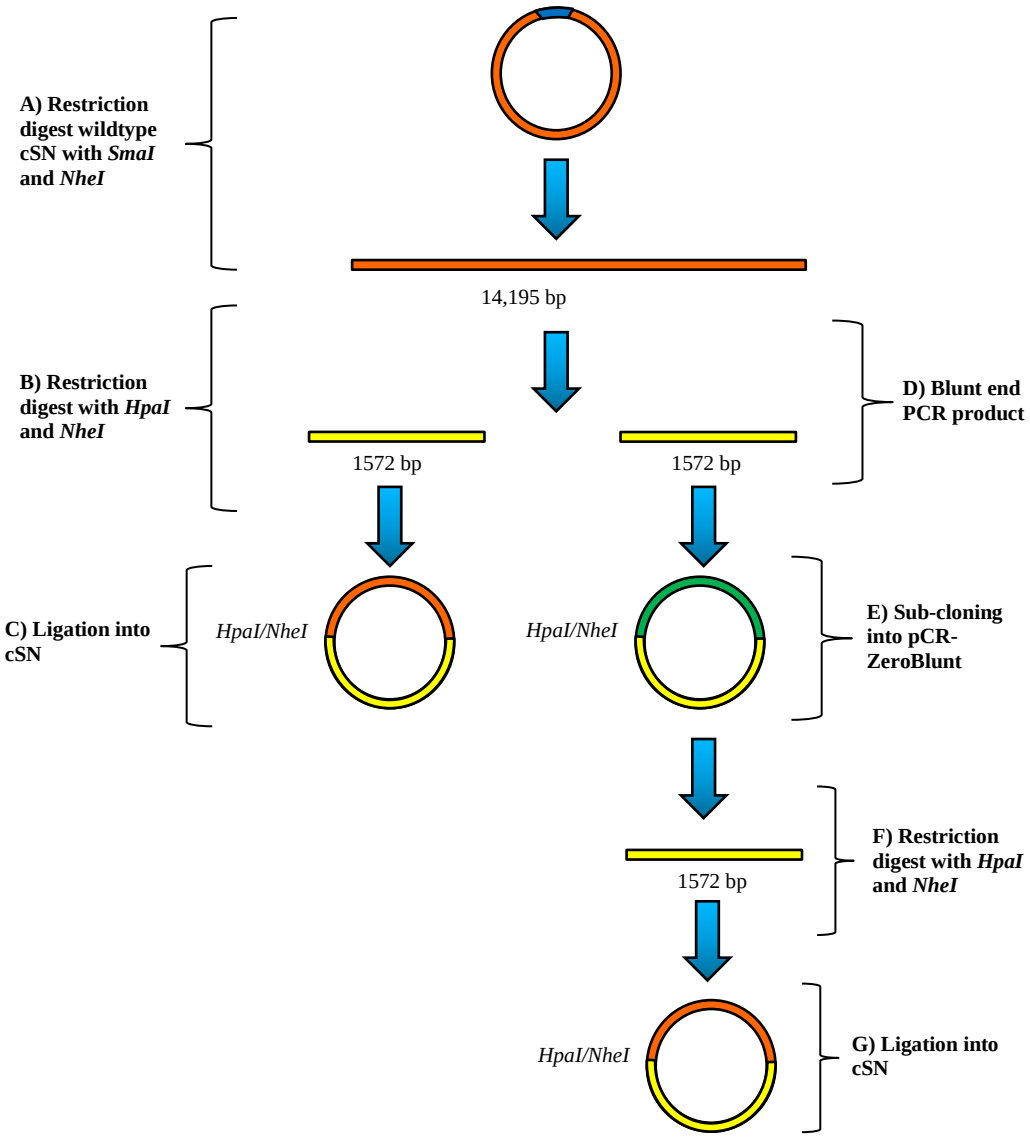


Figure 7.2: Cloning strategy for HpaI/NheI glycoprotein into full length clone. The strategy for cloning CVS, CVSFSS, WCBV and IKOV into cSN. The wildtype cSN glycoprotein is highlighted in blue, the cSN vector DNA is in orange, pCR-ZeroBlunt vector is in green and the glycoprotein insert is in yellow.

preparations were ligated into the pre-digested cSN vector. The ligation reactions were transformed into MAX Efficiency® DH5α-T1^{R} competent cells (Invitrogen) in the presence of the selective antibiotic, ampicillin. Small scale plasmid mini preparations were generated for putative positive clones and these sequences were confirmed by restriction enzyme mapping and sequence analysis. Plasmid DNA from confirmed positive small scale preparations was grown up into large scale preparations and the purity and concentration of the resulting preparations was measured on a spectrophotometer. Three positive maxi preparations from three separate positive colonies were generated for each full length clone. This strategy was successful in the generation of the CVS, CVSFSS, WCBV and IKOV full length clones however despite multiple attempts, the LBV and LBVFSS clones could not be correctly constructed.

In an attempt to generate full length clones containing LBV and LBVFSS via ligation an alternative blunt end restriction enzyme site was selected, *PmeI*. This restriction enzyme recognises an 8 nucleotide sequence, unlike *SmaI* and *HpaI* which both recognise a sequence of 6. This meant that two amino acids from the cSN backbone, within the intergenic region were converted into the first two residues of the *PmeI* site during the primer design, in order to avoid the addition of nucleotides. This new restriction enzyme site was engineered upstream of the LBV and LBVFSS G genes via PCR. These PCR products were then ligated into the subclone vector and the previous cloning strategy was then repeated. This alteration of the restriction enzyme sites showed no improvement in the generation of these clones so an alternative cloning strategy was then attempted.

Gibson Assembly (NEB) involves the generation of overlapping PCR products in order

to construct the clones (Figure 7.3). Primers were designed using an online tool provided by NEB (NEBuilder) to incorporate the *PmeI* and *NheI* sites up and downstream of the G genes. These primers overlapped with an additional set which would encompass the rest of the cSN backbone. PCR products successfully generated using these primers were subsequently incorporated into an assembly reaction and the results of these assemblies were transformed into NEB 5-α competent *E. coli* cells. Small scale plasmid preparations were generated for putative positive clones and the sequences of these were confirmed in the same manner as those by conventional cloning; restriction enzyme mapping and sequence analysis. Large scale plasmid maxi preparations were generated for positive clones and their purity and concentrations were determined. Again three maxi preparations were generated for each clone. This method of Gibson Assembly was able to generate the two remaining full length clones; LBV and LBVFSS in cSN.

7.3 Rescue of viruses from cDNA

The rescue of the recombinant full length clones using the reverse genetics technique was conducted as described in Section 2.5, see Figure 1.4. The purest plasmid maxi preparation of each full length clone was selected, along with the wildtype cSN full length clone as a positive control. These were transfected at a concentration of 0.3µg into FP-T7 infected BHK cells using FuGENE 6 (Promega) along with four helper plasmids: pN (0.8µg), pP (0.4µg), pG (0.4µg) and pL (0.4µg) in 24 well plates. This passage was designated as passage 1 (P1). After 3 days the cells and supernatants were harvested and transferred onto 50% confluent BHKs in 12 well plates. Due to the lack of visible cytopathic effect upon infection by lyssaviruses, an aliquot of 200µl from each of the freshly seeded 12 well plates was transferred into 96 well plates, in duplicate.

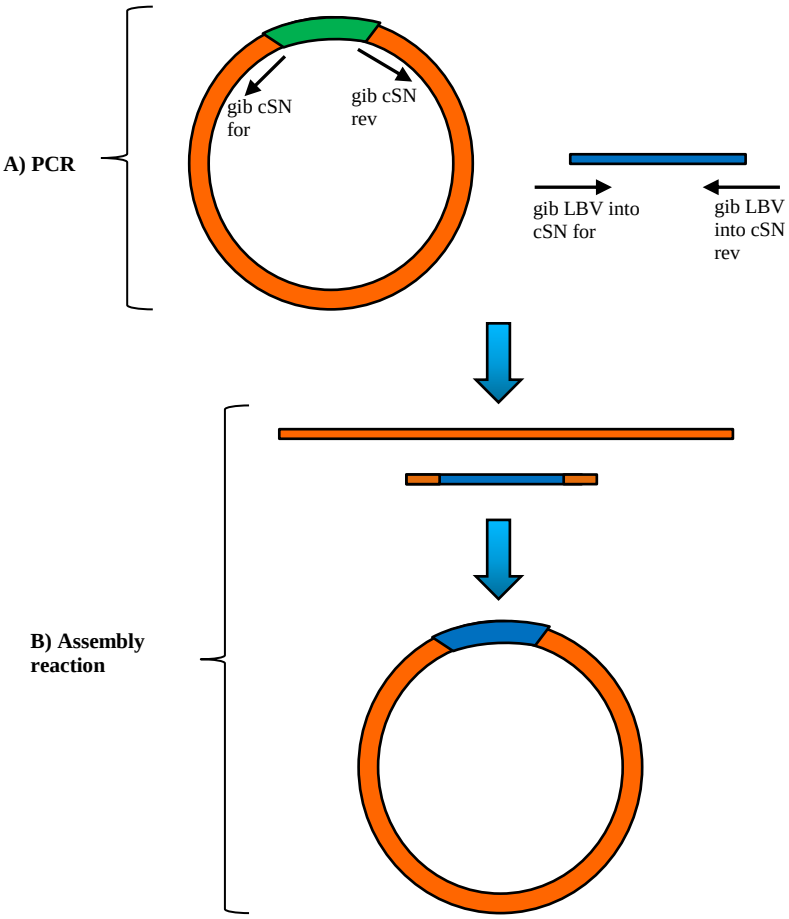


Figure 7.3: Cloning strategy via Gibson Assembly (NEB) for glycoprotein into full length clone. The strategy for cloning LBV and LBVFSS into cSN. The wildtype cSN glycoprotein is highlighted in green, the cSN vector DNA is in orange, and the glycoprotein insert is in blue.

Following a further 72 hour incubation; P2, the 96 well plates were fixed in 80% acetone then subsequently stained with an anti-N FITC conjugated monoclonal antibody (Fujirebio) to assess the proportion of cells that were positive for antigen. All of the rescues at this stage showed a number of positive cells. These were cells showing bright apple green, perinuclear granular fluorescence. The P2 cells and supernatant from the 12 well plates were transferred onto 50% confluent BHKs in 6 well plates. Aliquots into 96 well plates were again carried out. After 72 hours incubation; P3, the 96 well plates were fixed and stained. At P3 the CVS rescues suggested approximately 100% cells were positive for antigen. The WCBV cells were approximately 70% and IKOV approximately 30% positive. The LBVFSS wells showed a number of positive cells whereas the CVSFSS and LBV wells showed only a small number of positive cells and no visible foci of infection could be detected. In order to increase the volume of each virus, the cells and supernatants from the 6 well plates were transferred onto 50% confluent BHKs in T25 flasks. After a further 72 hour passage, designated P4, cSN-CVS was 100% positive for antigen, cSN-LBVFSS showed multiple positive cells but no foci of infection, cSN-LBV and cSN-CVSFSS showed a limited number of positive cells but no foci and cSN-WCBV and cSN-IKOV both showed multiple foci of infection. The cells and supernatants were split in half across two fresh T75 flasks and these were incubated for 72 hours. After the incubation the flasks were frozen at -80°C in order to lyse the cells to release the maximum yield of virus. The flasks were subsequently thawed and the cells and supernatants were separated by centrifugation. The supernatants of each virus rescue were inoculated onto 50% confluent BHKs in T75 flasks and RNA was extracted from the remaining cell pellets. In order to confirm the correct sequence of each of the rescues at this fifth passage the RNA was used to generate cDNA which was then used as a template in G specific KOD PCRs. The

resulting products were all of the expected size; 1.5 kb so were subsequently sequenced with G specific primers. The correct sequence of each rescue was obtained, confirming that no contamination had occurred.

Due to the low level of cells positive for antigen in the cSN-CVSFSS and cSN-LBV rescue wells at P3-5, a repeat transfection; P1 was set up with alternative full length clone maxipreps of both cSN-CVSFSS and cSN-LBV alongside cSN-CVS as a positive control. These were blind passaged to P2 at which point the corresponding 96 well plate was fixed and stained to detect fluorescence. Again very few cells were positive for antigen but despite this a further three passages were conducted, up to P5, however the number of positive cells at each passage consistently failed to increase, suggesting that the growth/viability of these viruses was severely restricted. A subsequent rescue attempt with passage up to P3 was also unsuccessful. In a final attempt to generate viruses from these plasmids some supernatant from the initial P1 rescue attempt was inoculated onto a fresh batch of BHK cells. The cells and supernatants were passaged up to P4 where cSN-CVSFSS again consistently failed to produce any more than 10 positive cells. At this point the decision was made to stop any further attempts to generate cSN-CVSFSS due to time constraints. In contrast cSN-LBV at P4 showed a cluster of positive cells so passage of cSN-LBV was continued. At P6 one single focus of infection was visible. The cells and supernatant were split into thirds across three fresh T75 flasks and after 72 hours incubation these P7 cells showed 10 separate foci of infection. It was expected that P8 would show 100% of cells positive however only one focus of infection was present. For this reason an aliquot from P7 that had been stored at -80°C was used to inoculate fresh cells in a repeat of the previous P8 passage however after 72 hours incubation this passage also failed to produce multiple foci of infection.

The cells and supernatants were passaged up to P10 without the presence of any more than one focus of infection so due to time constraints further passage or rescue attempts of cSN-LBV were abandoned.

In contrast, the cSN-CVS showed 100% cells positive for antigen at P5 so after a freeze thaw cycle the cells and supernatant were separated by centrifugation. The supernatant was then stored in 1ml aliquots at -80°C prior to titration. The remaining viruses were further passaged until all reached 100% positive cells. cSN-IKOV reached 100% at P6, cSN-WCBV at P7 and cSN-LBVFSS at P9.

Once all viable viruses had been generated and the presence of N protein had been confirmed at each passage the viruses were inoculated onto BHK cells which are highly permissive for lyssavirus infection. After 72 hours incubation the cells were fixed with PFA, permeabilised with triton X-100 and the cells were stained for actin-phalloidin, nuclei and glycoprotein using a rabies glycoprotein specific mAb (MyBioSource). A representative image of immunofluorescence staining is shown in Figure 7.4.

7.4 Titration of recombinant viruses

Once all four viable recombinant viruses had been successfully grown to 100% infection the supernatants were titrated. Alongside the recombinants a sample of wildtype cSN was also titrated. The titres achieved are shown in Table 7.1.

The titre of wildtype cSN was the highest at one log greater than cSN-WCBV. cSN-IKOV had a titre similar to that of cSN-WCBV whereas cSN-CVS was considerably lower. This was unexpected as cSN-CVS appeared to grow faster than cSN-WCBV and

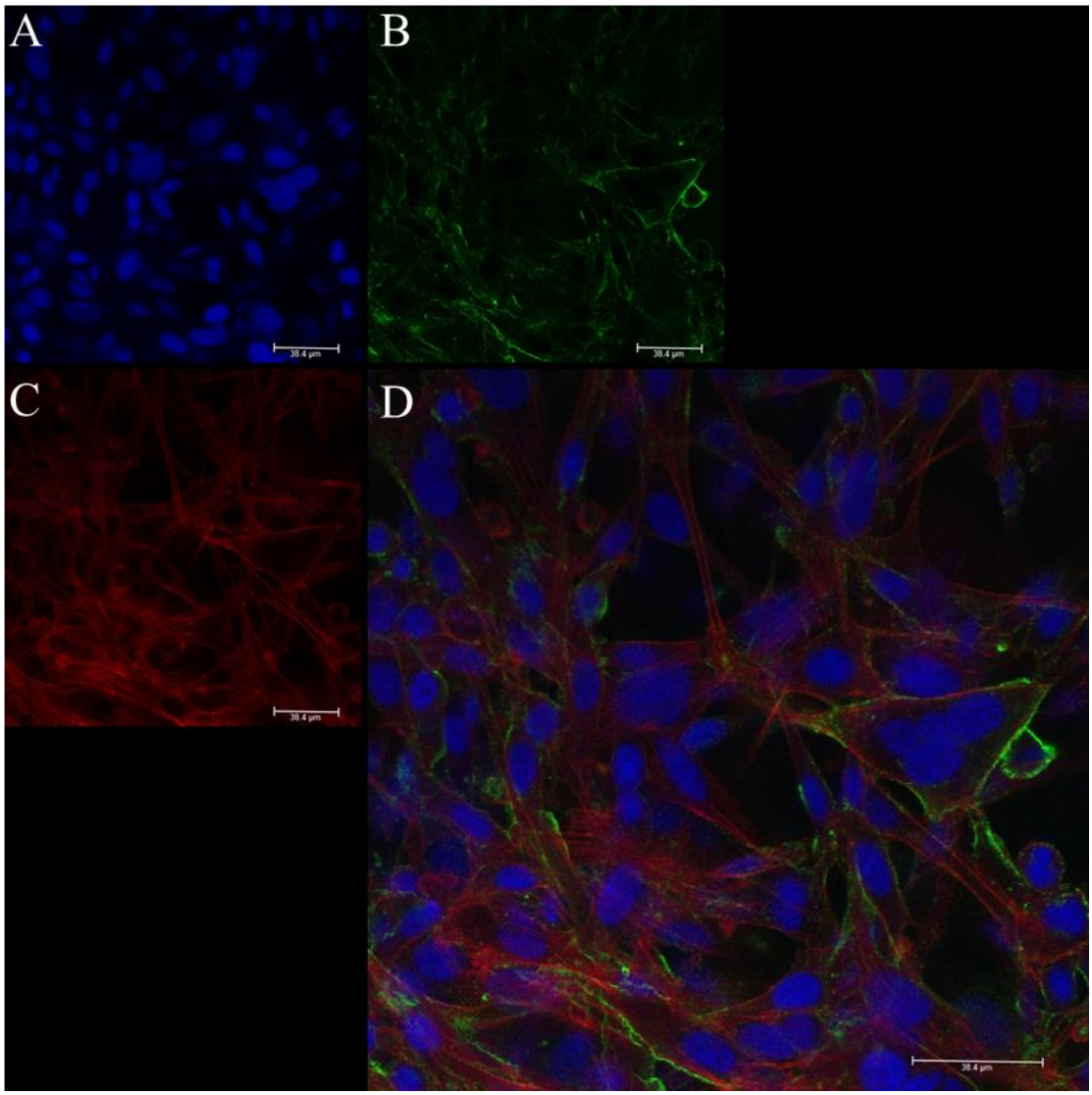


Figure 7.4: Assessment of glycoprotein distribution in recombinant cSN-IKOV virus infected cells. (A) Nuclei are stained blue; DAPI (VectaShield); (B) the RABV G protein is stained with Alexa Fluor 488 conjugate (Green); (C) the actin cytoskeleton is stained red (phalloidin conjugate-568); (D) and the merged images indicate that the glycoprotein (G) is present both associated with the endoplasmic reticulum/Golgi in the perinuclear region of the cytoplasm as well as being present on the cell plasma membrane.

Table 7.1: Titres of rescued recombinant viruses and wildtype cSN.

Virus	Titre (ffu/ml)
cSN	$3.3x10^6$
cSN-CVS	$8.5x10^4$
cSN-LBVFSS	$4.0x10^3$
cSN-WCBV	$3.3x10^5$
cSN-IKOV	$1.3x10^5$

cSN-IKOV. The lowest titre was that of cSN-LBVFSS and this mirrored the time taken for this recombinant to reach 100% infection as it required 9 passages to reach this point.

7.5 Growth kinetics of recombinant viruses

The titres of each of the rescued viruses had now been calculated so the growth kinetics of these could now be determined *in vitro*. Due to the low titres of both cSN-CVS and cSN-LBVFSS a single step growth curve was not possible to conduct for all viruses so a multiple step growth curve using a multiplicity of infection (MOI) of 0.01 was performed.

The growth kinetics of the viruses can be seen in Figure 7.5. There was a striking difference between the growth curve of wildtype cSN and the recombinant viruses (Figure 7.5 A). cSN did not start at zero ffu/ml but grew to a peak titre of $8.35x10^8$ ffu/ml at 72 hours post infection (hpi) whereas the peak titre of the next most successful virus; cSN-WCBV was $2.92x10^5$ ffu/ml at 96 hpi. All viruses except cSN-LBVFSS were detectable by 18 hpi. cSN-LBVFSS was detected from 24 hpi at a very low level of $4.85x10^1$ ffu/ml until it disappeared by 96 hpi. The end point titre of cSN-IKOV was $5.33x10^4$ ffu/ml which is slightly lower than that of cSN-WCBV whereas cSN-CVS reached an end point of just $1.17x10^3$ ffu/ml after gradually reducing from its peak titre of $5.84x10^3$ ffu/ml at 24 hpi.

The patterns of growth of each of the viruses also differed. It appeared that the growth of cSN was reducing by 96 hpi as its titre had slightly reduced from 72 hpi. Likewise cSN-CVS appeared unable to maintain a productive infection as its titre consistently decreased after 24 hpi. In contrast cSN-WCBV and cSN-IKOV both appeared to be

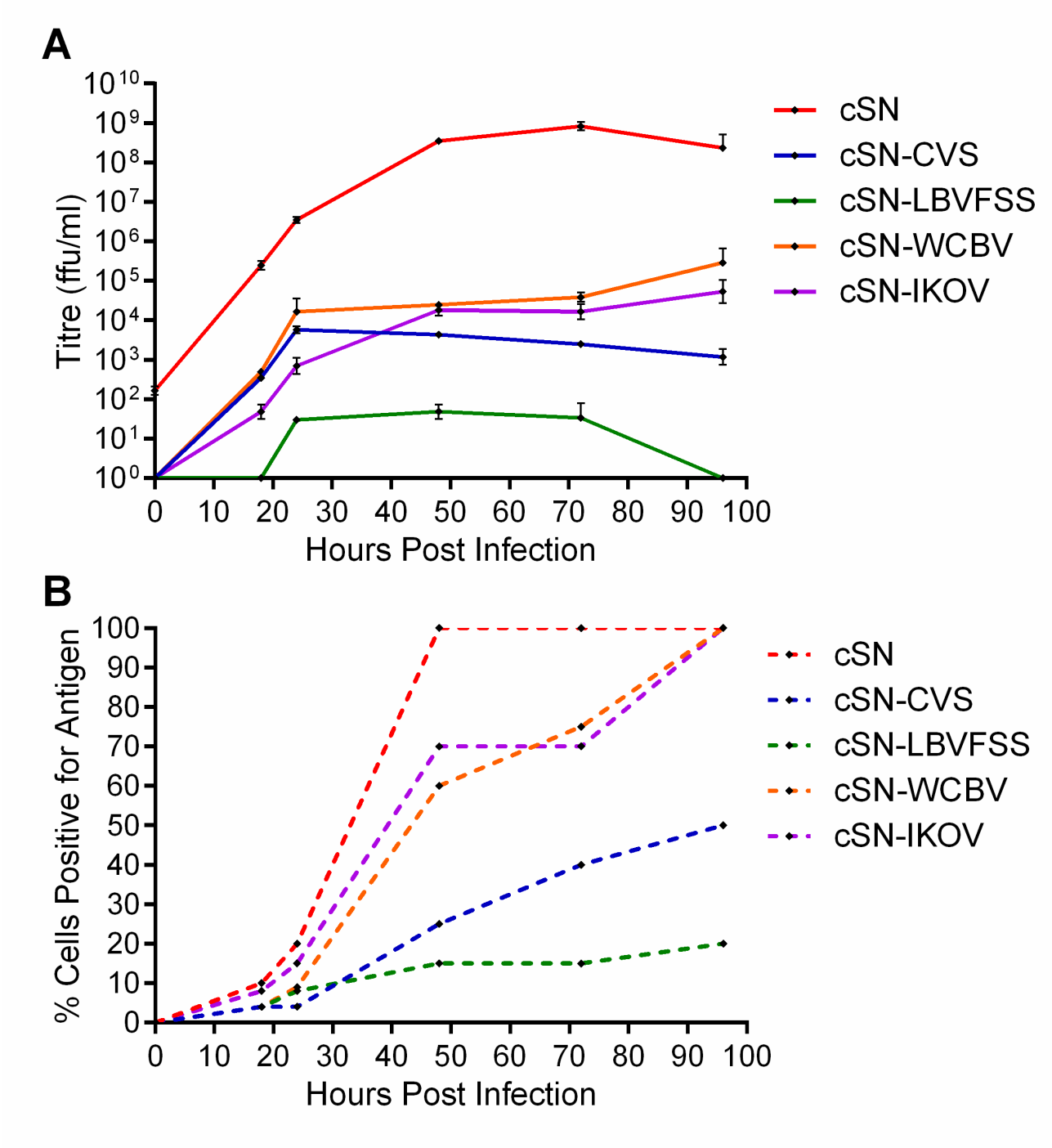


Figure 7.5: Growth kinetics of recombinant lyssaviruses *in vitro*. (A) Multiple step growth curves of each virus starting from MOI 0.01. The time course was undertaken twice, at each time point virus titres were determined in triplicate on BHK cells. The mean of the combined results are displayed. The error bars represent the SD about the mean of each titre. (B) Proportion of cells positive for antigen. cSN, cSN-WCBV and cSN-IKOV reached 100%, unlike cSN-CVS and cSN-LBVFSS.

increasing in titre over time as both peaked at 96 hpi. This suggests that both of these viruses grow at a slower rate than the wildtype. Aliquots of culture supernatants were collected at the designated time points and virus titres were determined on BHK cells in triplicate. The time course was undertaken in duplicate for all five viruses.

The trend of each virus is replicated in the percentage of cells positive for antigen at each time point (Figure 7.5 (B)). cSN-LBVFSS had the lowest number of antigen positive cells at each time point however the proportion of positive cells increased slowly to a peak at 96 hpi, unlike the titre of virus in the supernatant. Likewise, though cSN-CVS only reached an end point of 50% positive, the proportion of positive cells steadily increased from 0 hpi, unlike the titre in the supernatant which decreased from 24 hpi. cSN, cSN-WCBV and cSN-IKOV all reached 100% positivity with cSN at 48 hpi and the recombinants at 96 hpi. Although cSN-WCBV and cSN-IKOV both reached 100% positivity by the end time point neither reach a titre equal to that of cSN which was also 100% positive by the final time point.

The titres of each of the viruses (Table 7.1) indicated the trend seen in the growth kinetics experimentation. cSN had the highest titre; and grew to the highest titre in the growth curve and reached 100% positivity before any other virus. cSN-WCBV and cSN-IKOV both had similar titres and showed very similar curves as well as % cells positive at each time point. cSN-CVS had a lower titre than the previous two recombinants and this was reflected in its growth kinetics, just as cSN-LBVFSS had the lowest titre and failed to establish a productive infection. This suggests the efficiency of cell to cell spread of the recombinant viruses was reduced in comparison to cSN. This also lends support to these findings as the trends remain the same in each experiment.

7.6 In vivo assessment of recombinant viruses

7.6.1Vaccination of mice prior to intra cranial (i.c.) challenge

In order to determine the degree of protection afforded by available vaccines against the recombinant viruses, a group of mice were vaccinated with a current human rabies vaccine; Rabipur (Novartis). Mice were vaccinated via the intra peritoneal (i.p.) route with 500µl of a 1 in 20 dilution of vaccine (approximately 0.06 IU in 500µl) on day 0 and then again, with the same vaccine dose on day 14. On day 21 post vaccination blood samples were collected via tail bleeds and sera were isolated (Section 2.10.2.4). Each serum sample was run on a partial dilution series PNA (Section 2.3.3); starting at a 1 in 20 dilution, due to the very low volume of serum obtained from a mouse tail bleed, finishing at a 1 in 640 dilution. This enabled determination of whether the mice had seroconverted to a titre comparable to the WHO standard 0.5 IU/ml serum. The sera from the two groups of mock vaccinated mice were pooled and all serum samples from vaccinated mice were titrated individually, except for three individuals where a sufficient volume of serum for input into the PNA was not obtained.

The WHO control serum sample had a reciprocal titre of 639.99. The mock vaccinated pool 1 had a titre of 79.99 and the mock vaccinated pool 2 had a titre of just 11.31 indicating that none of these mock vaccinated mice had seroconverted. Four mice seroconverted to the same titre as the WHO control; 639.99 and 51 of the mice seroconverted so strongly that there was neutralisation to the end of the scale suggesting that these 57 mice had titres greater than 905.09. Two of the vaccinated mice did not seroconvert to the WHO control level, both reached just 22.62. Unfortunately there were insufficient sera remaining to repeat these two samples however their microchip numbers were noted.

7.6.2 Survival of vaccinated mice challenged i.c. with virus

Once the seroconversion of the vaccinated mice had been confirmed, all groups, both vaccinated and mock vaccinated were challenged i.c. with 100 ffu/ml of each virus in 30µl. Ten vaccinated mice and 5 mock vaccinated mice were challenged with each virus. The viruses inoculated were: cSN, cSN-CVS, cSN-LBVFSS, cSN-WCBV, cSN-IKOV and an isolate of LBV lineage B virus due to the lack of recombinant cSN-LBV. All mice were challenged 28 days post initial vaccination.

Mice were left until 21 days post challenge then survivors were terminated and cardiac bleeds taken. The survival curves of the mock vaccinated and vaccinated mice are shown in Figure 7.6.

7.6.3 Pathogenicity of recombinant lyssaviruses

In addition to the vaccination/challenge experiment a study into the pathogenicity of each of the recombinant viruses in naïve mice was conducted. Three to four week old mice were peripherally inoculated into the footpad (f.p.) (Section 2.10.3.2).

These mice were monitored for 21 days post peripheral inoculation, with clinical assessment twice daily. Any mice reaching clinical score 3 (Appendix III) were terminated. The pathogenicity of the recombinant viruses and LBV was then compared. The survival curve to 21 dpi data is shown in Figure 7.7.

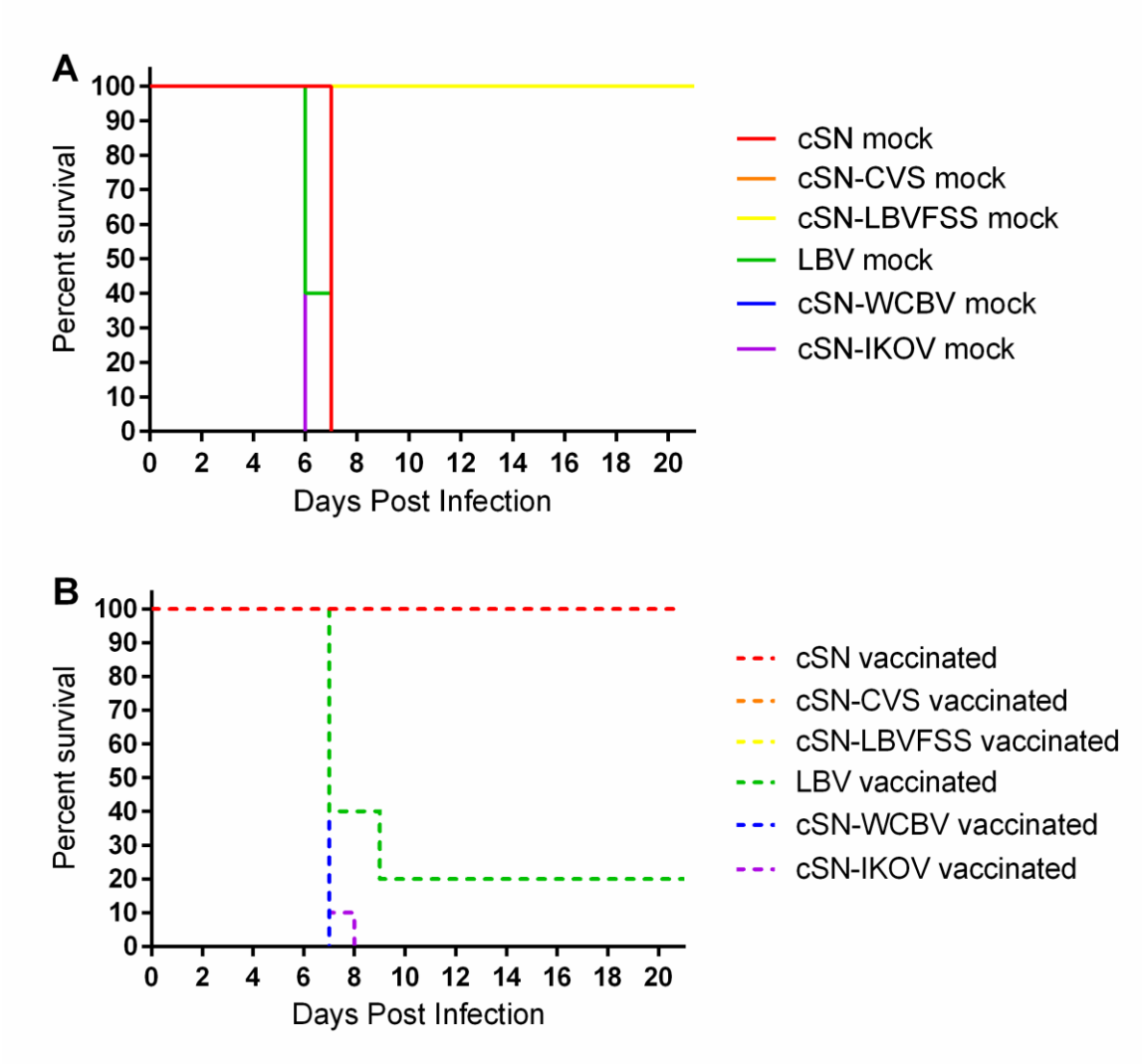


Figure 7.6: Survival curve of mice challenged i.c. (A) The survival curve to day 21 post infection of mock vaccinated mice n = 5 per virus. (B) The survival curve to day 21 post infection of mice vaccinated with Rabipur n = 10 per virus. Each mouse was challenged with 100 ffu/30µl of virus via the intra cranial route.

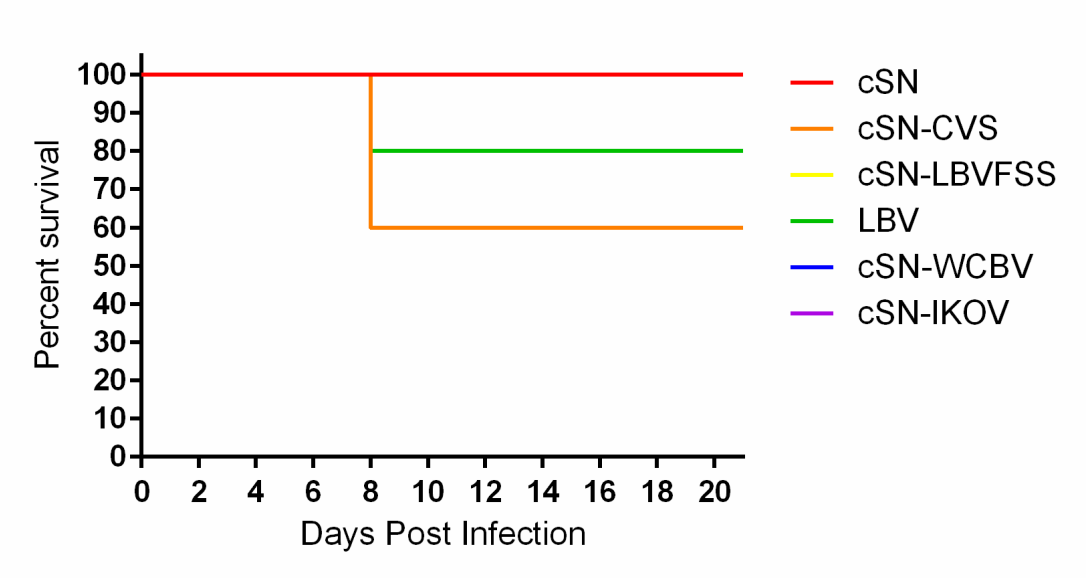


Figure 7.7: Survival curve of mice challenged peripherally. Each mouse was challenged with 1000 ffu/50µl of virus via the foot pad, except the low titre cSN-LBVFSS which was inoculated neat at 200 ffu/50µl. n = 5 per virus.

The survival curves of mock vaccinated mice challenged i.c with virus (Figure 7.6 (A)) showed that all of the mock vaccinated mice died by day 7 with the median survival of cSN-CVS, LBV and cSN-WCBV at 6 dpi and cSN and cSN-IKOV at 7 dpi. All of the cSN-LVFSS mock vaccinated mice survived with no clinical signs at day 21.

The survival of mice vaccinated with Rabipur (Figure 7.6 (B)) showed all mice challenged with a virus containing a phylogroup I G protein had survived to 21 dpi alongside the cSN-LBVFSS challenged mice which also all survived to day 21. In contrast 20% of the mice challenged with LBV had survived to day 21 whereas all those mice challenged with cSN-WCBV and cSN-IKOV had died by 7 and 8 dpi respectively.

The mice challenged peripherally by the six viruses showed a different trend to those inoculated intra cranially. On day 8 dpi 40% of the cSN-CVS challenged mice had died and 20% of both LBV and cSN-IKOV challenged mice had died. The remaining cSN-CVS, LBV and cSN-IKOV mice all survived to 21 dpi whereas all mice challenged with cSN, cSN-LBVFSS and cSN-WCBV survived to 21 dpi.

7.7 Discussion

A panel of antigenic site swap and divergent lyssavirus glycoproteins in a vaccine strain backbone has successfully been generated. Despite correct plasmid sequences and multiple rescue attempts only four of the six clones could be rescued successfully. The growth kinetics of these viable viruses was investigated with the aim of using these viruses to challenge vaccinated mice post vaccination.

Two plasmids consistently failed to produce viable virus; cSN-CVSFSS and cSN-LBV. The failure of cSN-CVFSS to rescue or grow to a detectable level mimics the finding

from the PNA as this glycoprotein failed to produce sufficient PT for use in PNAs. This suggests that the mutations made to the antigenic sites have irreparably affected the functionality of this glycoprotein. There may have been substantial alteration to the folding of the CVSFSS G or alterations in its glycosylation pattern which reduced or blocked its ability to form viable viruses and PTs. The failure of cSN-LBV to rescue was not predicted from the PT data as the LBV PT grew to consistently high titres. In an attempt to determine the cause of the failures of these two viruses, a structural model of the CVS and LBV wildtype glycoproteins as well as the antigenic site swap mutants in both monomeric and trimeric form was predicted, from a published structure.

To enable this SWISS-MODEL was utilised to identify any existing viral structures that shared identity with lyssavirus sequences. The model identified was the prefusion form of the VSV spike glycoprotein (Roche *et al.*, 2006). VSV G shares 27.5% identity with CVS and 31.0% identity with LBV at the amino acid level (AlignX). This level of identity is low to use as a template however in the absence of any lyssavirus G structural data there is no available alternative. Template-target alignments were generated in SWISS-MODEL followed by a predicted model from the resulting alignments. A monomeric model was automatically generated in preference to the trimeric form due to the lack of sequence identity; however a trimeric form was then generated, despite the SWISS-MODEL prediction. The trimeric models all had substantially lower Global Model Quality Estimation (GMQE) scores than the monomeric models signifying their lower accuracy however; as all of these models are predictions their accuracy cannot be improved without a more suitable model as a template. Once the models had been generated in SWISS-MODEL they were manipulated in PyMOL (version 1.3r1) in order to highlight the areas of the protein of most interest to this investigation. The monomeric

form of the lyssavirus G is shown in Figure 7.8. The antigenic sites are depicted in the surface conformation in order to highlight the area of each site which is expressed on the surface of the protein. The remainder of the protein is depicted in the stick form. From the top view of each monomer, antigenic sites I, IIa, IIb and III are visible on the CVS and CVSFSS models. For LBV and LBVFSS the models differ as antigenic site IIa appears to be almost entirely concealed by site IIb, i.e. there is much less of the IIa epitope present on the surface of the LBV and LBVFSS glycoproteins than CVS and CVSFSS. This may be interpreted in several ways. Firstly, this may suggest that the alteration of the antigenic site sequences is not sufficient to drastically alter the location of the antigenic sites on the mature protein as the patterns on the surface of CVS and CVSFSS remain similar, despite the sequences differing and the same pattern is evident with the LBV and LBVFSS models. Secondly, this alteration in the accessibility of antigenic sites may indicate that the positioning of antigenic sites on the G surface, and any masking of antigenic sites through mutation, may affect the importance each site plays in a cross neutralising response. From the PNA data with the single and full antigenic sites swaps in Chapter 5 it was found that sites I and III were important in a phylogroup I neutralising response and site II was important in a phylogroup II neutralising response. This may be related to the conformation of the sites as site I is displayed in a fairly similar conformation on all models in Figure 7.8. However site III differs between models predicting the wildtype CVS and Gs containing mutations with G proteins containing a phylogroup I site III (e.g. CVS and LBV FSS) having fewer site III residues exposed on the surface than those containing the phylogroup II site III. This may be significant in the recognition of this epitope. Indeed, from the site III specific D1

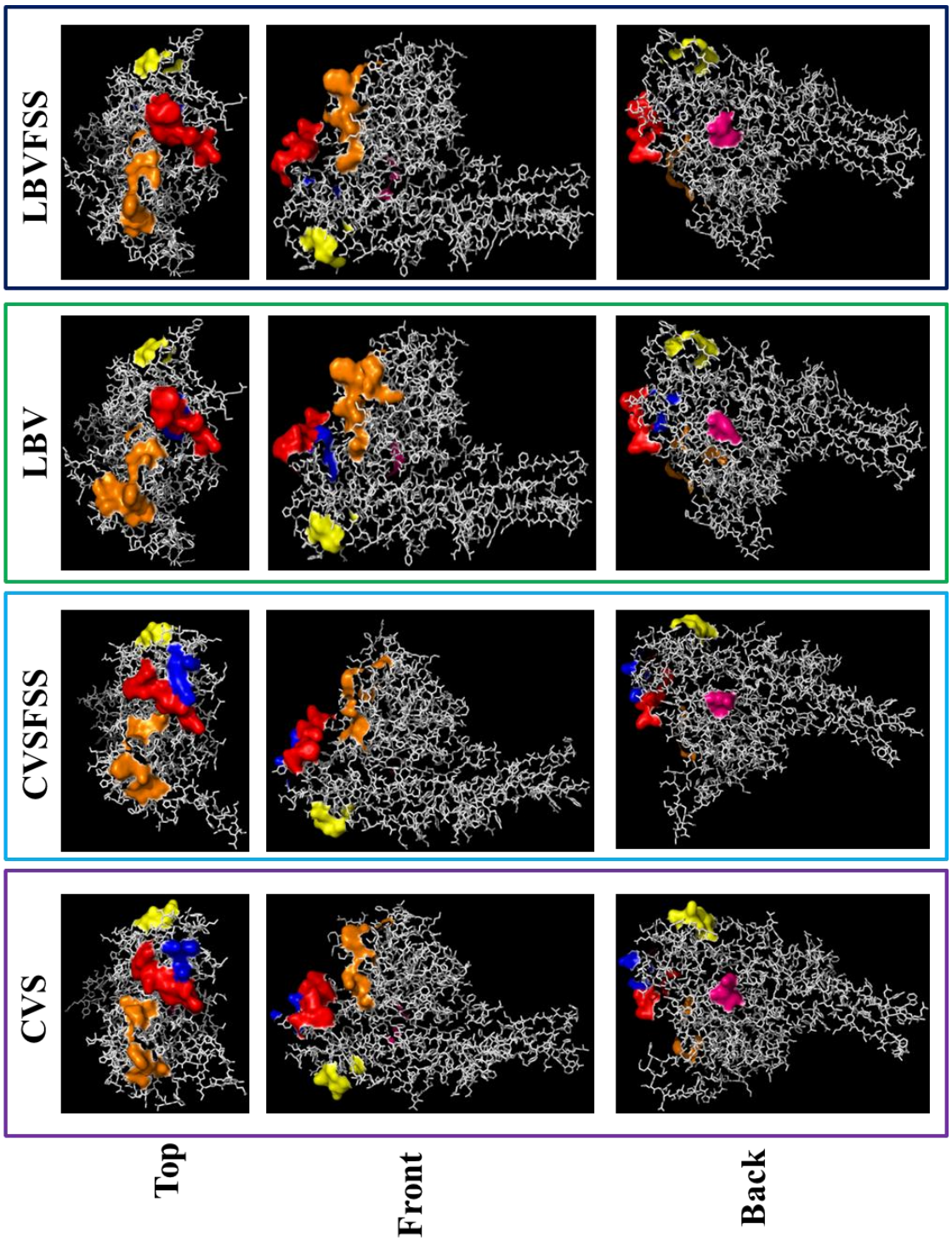


Figure 7.8: Model of monomeric lyssavirus glycoproteins. Antigenic site I is in yellow, antigenic site IIa is in blue, antigenic site IIb is in red, antigenic site III is in orange and antigenic site IV is in pink. The glycoprotein has been modelled using the prefusion VSV G (Indiana strain) as a template on the SWISS-Model server. The resulting model was subsequently manipulated and images created in PyMOL. The glycoprotein of each lyssavirus is shown in the same orientation to enable direct comparison. All four are shown as viewed from above, from the front of the model (as displayed in both modelling programmes) and from the back so that all 4 antigenic epitopes are visible.

mAb data in Chapter 6 it was evident that this site specific mAb was only able to neutralise wildtype CVS, it failed to neutralise LBVFSS suggesting that the conformation of this epitope, not simply the correct sequence, plays an essential role in recognition of antigenic site III.

The suggestion that antigenic site II may play an important role in phylogroup II neutralisation may be related to its conformation on the glycoprotein surface, as site II is displayed in a very different conformation on LBV backbone models than those generated with a CVS backbone. Site IIa appears to be less visible on the protein surface in phylogroup II models which may suggest that site IIb is in fact the immunodominant epitope. This is supported by the data from the CVS to LBV PNA (Figure 5.2) which shows that CVS to LBV IIb was more strongly neutralised by the LBV specific serum than any other construct. CVS to LBV IIaIIb was next most strongly neutralised followed by CVS to LBV IIa. All of these results together with the prediction model in Figure 7.6 suggest that site IIb, and or the interaction between site IIa and IIb would form an essential component of a phylogroup II neutralising construct.

The pattern of antigenic sites displayed on the surface of the trimeric form (Figure 7.9) of the lyssavirus glycoprotein models differs from that of the monomer. However the reduced accuracy of the trimeric model cannot be ignored when attempting to interpret this trimeric structure. The positioning of site IIa and IIb on the predicted CVS and LBV monomeric structures is no longer valid on the trimeric models for either G and as such it is difficult to interpret this data with respect to the potential roles of antigenic sites in virus neutralisation. In the absence of a more suitable structure, when examining the predicted trimeric model it is clear that antigenic sites I, II and III are all present on the

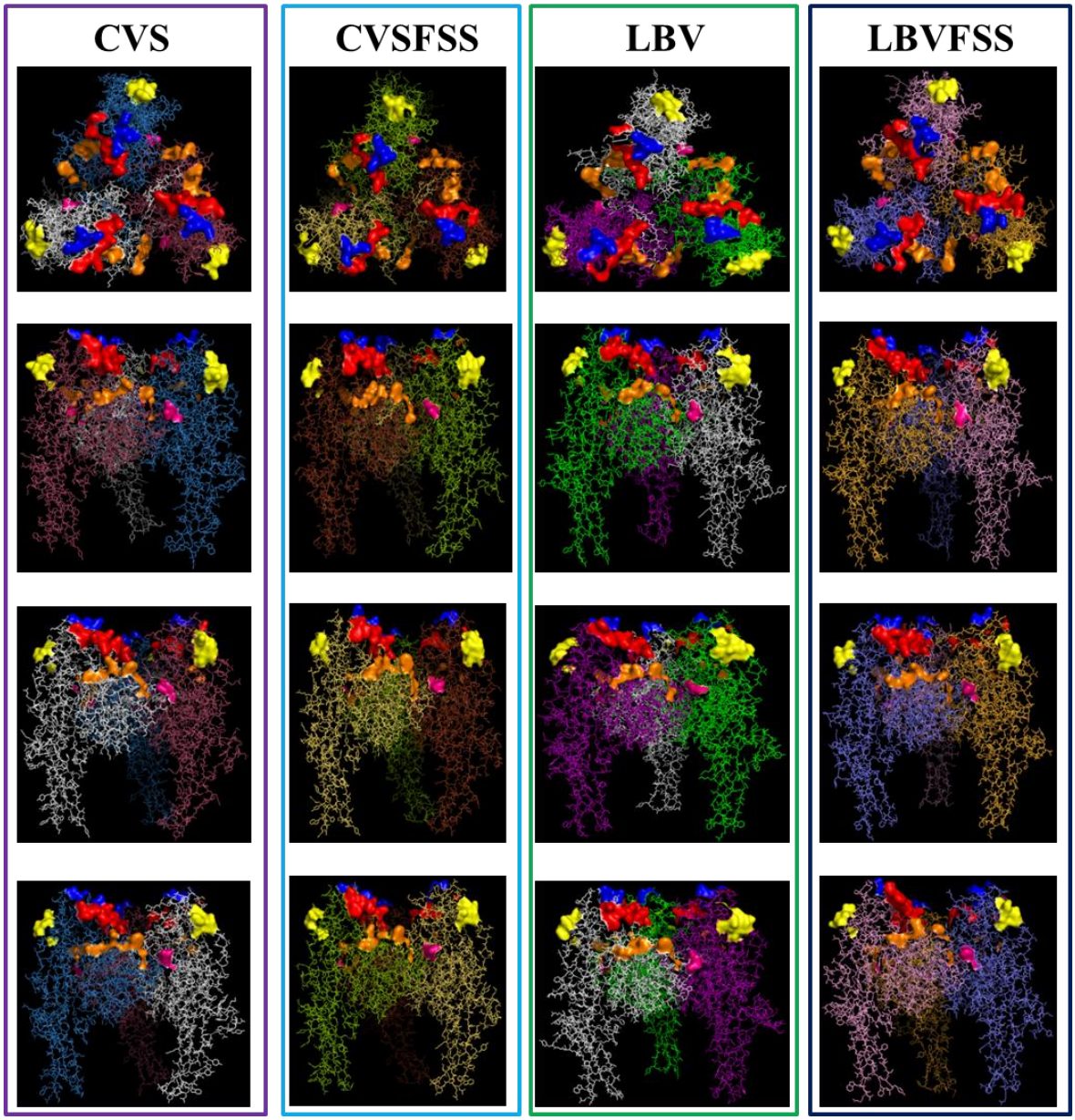


Figure 7.9: Model of trimeric lyssavirus glycoproteins. Antigenic site I is in yellow, antigenic site IIa is in blue, antigenic site IIb is in red, antigenic site III is in orange and antigenic site IV is in pink. The glycoprotein has been modelled using the prefusion VSV G (Indiana strain) as a template on the SWISS-Model server. The resulting model was subsequently manipulated and images created in pyMOL. The glycoprotein of each lyssavirus is shown in the same orientation to enable direct comparison. All four are shown as viewed from above, and from the front so that the interaction between each chain is visible.

tip of the ectodomain and access to these sites appears relatively unrestricted by the trimeric conformation. This may explain why these three sites play the most important roles in neutralisation. In contrast, though antigenic site IV is present on the surface, access to this site may be restricted by its position in the groove between two chains of the trimer. This location may inhibit access of many antibodies to this site, thus reducing its impact in the neutralising response. Antigenic site III is located in closest proximity to neighbouring chains which suggests it may be most negatively impacted by any alterations in trimerisation. This may explain the previous finding that site III specific mAb D1 was only capable of neutralising the CVS wildtype PT as folding of the trimer in the other PT preparations may have differed. In fact it has been proposed that D1 can only recognise the trimeric form of glycoprotein (Jallet *et al.*, 1999) which supports this finding as any alteration in the construction of the trimer would affect mAb binding.

Overall the models produced here provide limited insight into the location of antigenic sites on the lyssavirus glycoprotein however they have yielded some interesting explanations for previous findings of this investigation. The fact that sites IIa and IIb are in very close proximity to each other on the surface of these models lends support for some accuracy in this prediction as previous predictions have also placed the two distinct regions of this discontinuous epitope close together in the 3D structure of the glycoprotein. Until a crystal structure of RABV or any other lyssavirus G is resolved however any interpretations from this model remain limited (Dr Gaudin – personal communication). Despite this these predictions provide an opportunity to evaluate experimental findings.

The structural predictions have not provided significant insight into the reason for the

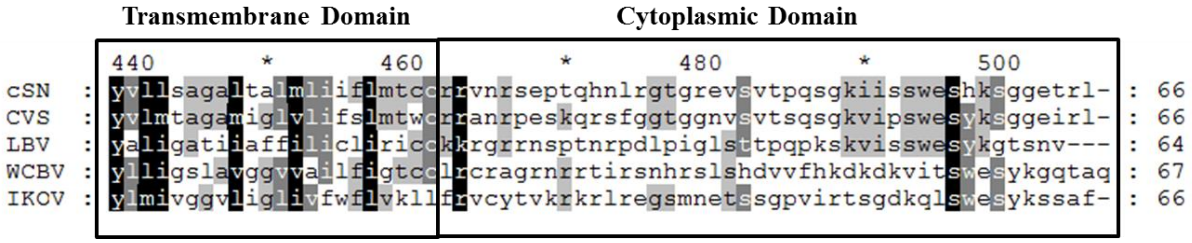
cSN-LBV virus rescue failures. However, one factor that may have affected virus rescue was the stability of this plasmid. As described previously cSN-LBV was problematic to generate, requiring alternative cloning techniques to construct the correct plasmid. It was hypothesised at the stage of bacterial transformation that the glycoprotein insert itself may have been toxic as bacteria transformed with the cSN-LBV ligations consistently failed to produce colonies post transformation. In addition, once Gibson Assembly had eventually yielded a correct plasmid, the maximum scale plasmid preparation had a lower DNA concentration than other full length plasmids which suggests that the bacteria were unable to grow to the usual capacity. This observation may therefore be applicable to the consistent failure of mammalian BHK cells to support production and infection of viruses containing this potentially toxic glycoprotein insert. In addition, throughout the numerous rescue attempts and repeated passages it was observed that BHK cells transfected to produce cSN-LBV and BHK cells exposed to TCSN from cSN-LBV transfected cells grew very poorly compared to cells infected with other recombinant viruses. This may again indicate a level of toxicity specific to this construct however due to time constraints a more detailed investigation into the cause of cSN-LBV failure was not possible.

The cSN growth curve did not start from zero ffu/ml suggesting either some input virus remained after washing or it was able to initiate a detectable infection after just one hour of adsorption. The other viruses that were rescued successfully all grew to titres 1 to 3 logs lower than the wildtype cSN. This suggests that the insertion of heterologous glycoproteins into the cSN backbone affects the growth capacity and cell to cell spread of the resulting viruses, most significantly for cSN-LBVFSS. This low titre correlates with the growth kinetics data in Figure 7.5 as cSN-LBVFSS had the slowest growth and

the lowest peak titre. It is possible that the mutations in the antigenic sites of the glycoprotein rescued its ability to grow as the wildtype cSN-LBV construct consistently failed. It may be that the presence of the CVS antigenic sites in the LBVFSS construct reduced the potential toxicity of the construct or rescued the folding or glycosylation of the protein (Chapter 8). cSN-CVS has the next lowest titre and end point titre on the growth curve. This was unexpected as CVS G has the closest homology to the cSN G so it was expected that this switch would have the least impact on the resulting virus. In fact the highly heterologous WCBV and IKOV G inserts produced higher titre viruses. This same pattern is seen in the PT titration data as CVS PT consistently grew to lower titres than WCBV or IKOV PTs. Within the context of a cSN based infectious virus the differing titres of these constructs may be a result of the interaction between the heterologous glycoprotein and the matrix protein to form the structure of the virus particle. The precise nature of the interaction between these two viral proteins remains undefined however it has been shown for a number of *Mononegavirales* including Respiratory Syncytial virus and VSV that M interacts directly with G to enable high efficiency viral budding from host cells (Enami & Enami, 1996; Ghildyal *et al.*, 2005; Lyles *et al.*, 1992). It has also been shown that the interaction between the rabies G and M proteins enables efficient budding of viral particles (Mebatsion *et al.*, 1999). It is the cytoplasmic domain of the G protein which has been shown to play the key role in this interaction (Genz *et al.*, 2012; Ghildyal *et al.*, 2005) so an alignment of the transmembrane and cytoplasmic domains of cSN and the other lyssavirus glycoproteins was made to look for sequence homology which might explain the success of some viruses over others. This can be seen in Figure 7.10.Certainly, if the titre of the viruses was directly related to the homology between the cSN and heterologous G it would be expected that cSN-CVS would have the highest titre of the recombinants, followed by

Figure 7.10: Alignment of glycoprotein transmembrane and cytoplasmic domains.

The alignment was made in GENEDOC. The wildtype G sequences used in construction of recombinant lyssaviruses are shown against the sequence of the backbone glycoprotein; cSN.



cSN-LBV. This is not the case which suggests that the homology between glycoprotein and matrix protein is not the sole determinant of successful virus rescue.

Despite cSN-WCBV and cSN-IKOV growing to higher titres than cSN-CVS and cSN-LBVFSS there was still difference between the growth kinetics of the recombinants and the wildtype cSN. It is clear that the replacement of the cSN G with a heterologous lyssavirus G, despite a close sequence homology between the wildtype and the inserted G, has a detrimental effect on the ability of the virus to grow and in some cases even inhibits the virus from initiating a productive infection. This is similar to findings from a previous study which incorporated the G proteins from EBLV-1 and EBLV-2 into cSN. This study found that both recombinant viruses grew to substantially lower end point and peak titres than cSN, however the titres of the recombinants were higher than those of the parental EBLV-1 and EBLV-2 wildtype viruses (Marston et al., 2013). This suggests that a further study into the growth kinetics of the recombinant viruses generated in this investigation in comparison with their wildtype virus counterparts could reveal the reason for the success of cSN-WCBV and cSN-IKOV over cSN-CVS as perhaps CVS virus grows to a lower titre in vitro than WCBV or IKOV. Although wildtype IKOV is available at APHA, WCBV is not so comparisons cannot be made.

The data from the survival curves indicated some substantial differences between survivorship in intra cranially challenged vaccinated and unvaccinated individuals. The cSN-CVS, LBV and cSN-IKOV viruses appeared to be most pathogenic when inoculated intra cranially into naïve individuals as these viruses had the shortest median time post infection to 0% survival. cSN and cSN-WCBV followed by just 1 day whereas all cSN-LBVFSS challenged mice remained healthy at the end of the experiment. This

pattern was conserved in the peripherally inoculated mice also as cSN-CVS, LBV and cSN-IKOV all showed 60-80% survival whereas all other viruses showed 100% survival by 21 dpi. This was reflected in the growth kinetics as cSN-LBVFSS grew at the slowest rate of all the recombinant viruses so it can be postulated that infection *in vivo* may also result in clinical signs days after those caused by other viruses. In addition, the vaccinated cSN-LBVFSS challenged mice were all healthy at day 21 but the role of vaccination in this result cannot be determined. In contrast there were clear differences between survival of vaccinated and unvaccinated mice challenged with other viruses; all unvaccinated mice challenged with cSN and cSN-CVS had died by 7 dpi whereas all vaccinated mice challenged with these viruses survived. This confirms published data (Brookes *et al.*, 2006) and data from Chapters 4 and 5 which describe strong cross neutralisation between phylogroup I viruses.

The survival of mice, vaccinated or unvaccinated with LBV, cSN-WCBV and cSN-IKOV also supports existing data describing a lack of protection against divergent lyssaviruses from vaccine induced immunity (Hanlon *et al.*, 2005). Both vaccinated and unvaccinated mice challenged with cSN-WCBV survived until 7 dpi and the same pattern was evident with the cSN-IKOV mice as 100% unvaccinated died on day 6 post infection and 90% of the vaccinated mice had died by 7dpi with the remaining 10% surviving just one more day to die on day 8 post infection. This confirms the total lack of protection afforded by rabies vaccines against these divergent lyssaviruses. Interestingly however 20% of the vaccinated mice challenged with LBV remained healthy at 21 dpi which suggested that the vaccine had induced a small degree of protection. This is reflected in a previous finding where dogs were protected against challenge with LBV after they had been vaccinated with Rabiffa vaccine. It would be

interesting to compare the sequences of the RABV strains used to generate both Rabiffa and Rabipur (used in this experiment). Additionally, data in Chapter 5 suggested that the WHO polyclonal serum from vaccinated humans was able to induce substantial (80-90%) neutralisation of LBV PT which may explain the survival of a small percentage of the LBV challenged mice, in addition these mice had very strongly seroconverted post vaccination. Interestingly the WHO serum is isolated from a pool of human sera, all having seroconverted to a human vaccine strain and the vaccine used in this experiment, Rabipur, is a human vaccine so this may suggest that some of the RABV strains used to generate human rabies vaccines have the capacity to generate a protective response in a small proportion of LBV B infections.

Chapter 8: Discussion

The utility of PTs in the investigation of the neutralisation profiles of dangerous zoonotic pathogens such as the lyssaviruses has been demonstrated. The panel of PTs enables investigation of the lyssavirus glycoprotein in situations where the live virus is unavailable and the presence of PT can be quickly identified by reporter gene readout, without the need for time consuming antibody based detection methods or the requirement of specialist containment facilities. However, at least for some lyssaviruses, including both wildtype and mutated glycoproteins, viable PTs could not be generated suggesting that the transfection process requires further optimisation. The variation in the rescue efficiencies and titres of PT viruses may be a result of differences in the quality of the plasmid preparations used to generate these constructs (Bouvrais, 2012).

The attempts to characterise some PT preparations suggested that there is great variability between PT particles in a population. The sizes of the PT particles appear to be larger, on average than virus particles and their greater range of sizes suggests a more pleiomorphic morphology than virus particles. In order to characterise lyssavirus PT particles it would be advantageous to be able to visualise them by electron microscopy in order to compare their morphology with that of lyssaviruses. It was also not possible to measure fluorescence of PTs directly using flow cytometry, despite the scatter of PTs being measurable. This was likely due to an alteration in the epitope of the mAb utilised in the flow cytometry experiment. In order to determine whether PTs can be directly measured an alternative mAb, also directed to the glycoprotein should be selected. This would enable an estimation of the concentration of G proteins expressed on the surface

of the PT population and also enable a qualitative estimation of the size differences between groups of particles in the population.

A downstream effect of the apparent variability within PT populations is a marked variation between replicates in the neutralisation assays. This variation was evident in the standard deviation around the mean percentage neutralisation of many PTs by the large range of sera tested. There are approximately 400 trimeric glycoprotein spikes on a rabies virion (Da Poian *et al.*, 2005) however the number of trimers on a PT particle is undefined. In order to increase the reliability of PNAs, the expression of glycoprotein trimers on PTs must be determined. This will enable a more accurate comparison between neutralisation data from both PTs and viruses.

A strong level of intra phylogroup I cross neutralisation was confirmed, in support of the current data from virus neutralisation studies and vaccine protection data (Brookes *et al.*, 2006; Hanlon *et al.*, 2005). The degree of cross neutralisation was found to be very strong with just 0.1 IU/ml hyperimmune sera strongly neutralising all phylogroup I PTs. Data from virus neutralisation assays defined a threshold of 0.5 IU/ml to be protective against lyssavirus infection however, in light of the 0.1 IU/ml finding from PNAs it would be of use to conduct a similar assay with live viruses in order to determine whether 0.1 IU/ml remains protective. This may impact on vaccination dosing regimens as current regimens require a series of vaccinations on days 7, 21 and 28 which requires a substantial level of commitment from patients and often these schedules are not adhered to (Sudarshan *et al.*, 2012; Wilde, 2007). If a lower titre of antibody is found to be protective against all viruses in phylogroup I, perhaps the number of doses in current

PEP regimens can be reduced which would provide effective protection to a greater proportion of patients and have substantial economic benefits.

There also appears to be a strong level of intra phylogroup II cross neutralisation with a marked lack of cross neutralisation with phylogroup III and IKOV. This degree of neutralisation within phylogroup II has been previously demonstrated (Badrane *et al.*, 2001) however this has not been investigated in such depth as neutralisation within phylogroup I. This investigation has highlighted the presence of a high degree of cross neutralisation of phylogroup II PTs with sera directed against LBV lineage B however further neutralisation assays using sera directed against the other members of phylogroup II would be beneficial to more accurately characterise the degree of neutralisation present.

Due to the high level of neutralisation within phylogroup I, as demonstrated by hyperimmune sera from both humans and dogs this suggests that the antigenic features of a RABV (as the backbone of all current vaccines) should be sufficient to induce antibodies capable of neutralising all other phylogroup I viruses. Likewise, due to the neutralisation of all phylogroup II PTs by LBV B sera, the antigenic features of the LBV B G protein should be sufficient to induce protective antibodies against all other phylogroup II viruses. A combination of the antigenic features of both CVS (a RABV) and LBV B should therefore be sufficient to induce protection against all viruses in both phylogroups I and II, however it is likely this construct would not be able to induce complete protection against viruses outside of these phylogroups. It therefore follows that a true pan-lyssavirus protective construct would contain antigenic features of WCBV and IKOV as well as CVS and LBV B. This is due to the divergence of WCBV

and IKOV away from CVS and LBV resulting in antibodies raised against these divergent viruses being unable to completely neutralise CVS and LBV B PTs and vice versa.

Now that one representative G protein from phylogroups I and II have been found to sufficiently represent all other members of their respective phylogroups the precise antigenic features of these glycoproteins which are responsible for the induction of protective immunity have potentially been defined. Previous studies into the antigenicity of the RABV G suggested that antigenic sites II and III were most immunogenic (Jallet *et al.*, 1999; Lafon *et al.*, 1983) and the results of this investigation confirm this as well as highlighting the role of antigenic site I in neutralisation. The models generated in Chapter 7 suggest that sites I, II and III are all present on the tip of the glycoprotein spikes so provide unrestricted access to antibody interactions. This goes some way to explaining the reason for the importance of these individual antigenic sites in a neutralising response however until a crystal structure is resolved; the speculation over the location and impact of the antigenic sites remains unsupported.

The antigenic site swap PNAs also highlighted variability in glycoprotein expression between different PT populations. It appeared that the LBV backbone PTs may express a lower concentration of G protein on the surface than the CVS PTs. This was due to the neutralisation profile of the LBV PT as it was strongly neutralised by 0.5 IU/ml RABV specific sera as well as 0.5 IU/ml LBV specific sera whereas the CVS wt PT was only neutralised by the 0.5 IU/ml RABV specific sera and not at all by the 0.5 IU/ml LBV specific sera. Upon dilution of the test sera 10-fold the neutralisation profile of the LBV PT was closer to the pattern expected from virus neutralisation data (David Selden –

unpublished data). This therefore lead to the hypothesis that there was less G present on the LBV PT which lead to its increased susceptibility to neutralisation by heterologous sera as a result of immunogenic epitopes outside of the antigenic sites. The reason for this reduction in the concentration of G on the LBV PTs is unknown and requires further investigation. A valuable experiment would utilise electron microscopy to gold label the glycoproteins on the surface of CVS wildtype PTs, LBV wildtype PTs and inactivated CVS and LBV viruses. This would enable visualisation of the viral and PT particles to compare morphology as well as determining any differences between the levels of G expression on the different particles. Alternatively, once a mAb is identified which is able to label PT for flow cytometry, this could be used to compare populations of PT and virus based on the mean fluorescence intensity of each population which is directly related to the amount of G expressed on the viral envelopes.

The reason for the apparent reduction in G expression on the LBV PTs is unknown however the link between this and the repeated failure of the cSN-LBV virus to grow may be significant. The defining feature of glycoproteins is glycosylation. The lyssavirus G proteins are N-linked glycosylated at asparagine (N) residues which are contained within specific glycosylation sequons. These sequons consist of asparagine-X-serine/threonine sequences where X can be any amino acid except proline (Shakin-Eshleman *et al.*, 1992).The RABVs contain three glycosylation sequons at asparagines 56, 266 and 338 (Shakin-Eshleman *et al.*, 1992). The LBV glycoprotein however lacks N 56 so contains only two glycosylation sites and amino acid 56 is within antigenic site IIb. It has been shown that N 56 is not efficiently glycosylated in the presence of complete sequons at positions 266 and 338 however in the absence of these sequons N 56 is sufficient for transport and expression of G on the cell membrane. The absence of

this glycosylation site in the LBV PT and the IIb mutants may impact the intracellular transport, antigenicity and the stability of the glycoprotein (Shakin-Eshleman *et al.*, 1992; Shakin-Eshleman *et al.*, 1993). As well as this it has been shown that regions of a protein downstream from sequons can influence their glycosylation (Shakin-Eshleman *et al.*, 1993) so the mutations in sites III and IV may have had some effect on glycosylation. These studies however were conducted in cell free systems so glycosylation may have been more or less effective than in cell based systems. In addition glycosylation may differ between cell types *in vitro* and efficiency likely differs *in vivo* also. This makes it difficult to define the precise effect of glycosylation but may explain the lack of G on the LBV PT and even the toxicity or failure of the cSN-LBV virus to rescue.

Progress has also been made into characterising the epitopes for a panel of apparently antigenic site specific mAbs. The antigenic site of mAb 62-7-13 had been previously characterised (Both *et al.*, 2013) and data from this investigation confirmed its specificity and the critical role of aa 245 in neutralisation. The epitope for E559 was less well defined however evidence from this investigation has highlighted the potential importance of leucine 57 as a critical residue for E559 neutralisation. The virus neutralisation data points towards a critical role for leucine 57 however the PNA did not provide such strong evidence. This was because the wildtype CVS PT was not strongly neutralised by E559 whereas live CVS virus is neutralised. The reason for the discrepancy here may be an altered display of G protein on the PT surface, as opposed to that found on the surface of a viral particle however this hypothesis requires further investigation, as previously described. In addition antigenic site II is the only known discontinuous conformational epitope on the lyssavirus glycoprotein which means it is

likely to be most susceptible to changes in folding or structure of the G protein, therefore any differences between the stability of the G protein in virus particles and PT particles (due to the lack of interaction with M protein) is likely to affect the conformation of antigenic site II and therefore may affect its neutralisation profile.

Despite the unexpected neutralisation profile of the wildtype CVS PT, the other site swap PTs were consistent with site IIb and therefore leucine 57 playing a role in E559 neutralisation. In order to investigate this hypothesis further by expanding on initial previous work (Muller *et al.*, 2009) the Kelev G protein should be incorporated into PT particles. This would enable site directed mutagenesis to generate the observed mutations at positions 169 and 170 to determine which mutation and which amino acid plays the most critical role in E559 neutralisation. These mutations should then be incorporated into Kelev virus glycoprotein and reverse genetics utilised to rescue these mutated viruses in order to confirm data from the PNAs. These future investigations may have substantial impact if key residue of the E559 epitope is defined as it is still under consideration by the WHO as a component of a potential therapeutic mAb cocktail (Both *et al.*, 2012; Muller *et al.*, 2009).

A panel of recombinant viruses was generated using reverse genetics techniques. However, some viruses rescued more successfully than others, likely due to differences in the heterologous glycoprotein substitutions. No cSN-CVSFSS virus could be generated from a full length genome plasmid containing the correct sequence for this recombinant. However, the PT expressing this G protein was also unsuccessful, perhaps because it lacked the glycosylation site at amino acid 56 which would normally be found in the wildtype CVS G protein. The cSN-LBV virus was also unsuccessful, again it

lacked the asparagine residue at position 56 which although didn't appear to affect PT generation so substantially, may have had a more detrimental impact in the context of the RABV cSN backbone as this would have contained N 56 in its wildtype G protein. It was surprising therefore that IKOV and WCBV rescued successfully and grew to higher titres than cSN-CVS as IKOV and WCBV do not have N56 whereas CVS does so it is likely some other factor is affecting the success of these recombinant viruses. It was found previously that a lack of success of a recombinant virus was linked to the success of the parental virus of the glycoprotein in question. cSN-EBLV-2 showed a marked reduction in growth although interestingly the parental wildtype EBLV-2 virus grew to an even lower titre (Marston *et al.*, 2013). This suggests that future work should be done to repeat the growth kinetics experiment in Chapter 7 alongside wildtype viruses where available including: CVS, WCBV & IKOV to determine whether the recombinant virus growth curves reflect the wildtype virus growth curves as this may help to explain the differences between the activities of the recombinant viruses generated in this investigation.

Clearly the utilisation of PTs and reverse genetics to study the neutralisation within the genus can help to understand the relationships between viruses within the genus and provide tools to further investigate the cross neutralisation afforded by responses to different glycoproteins. The lack of vaccine protection afforded by the current rabies vaccines against several members of the genus is of importance to both human and animal health. Frustratingly, whilst the data generated in this study are of interest, in the absence of a crystal structure for the rabies glycoprotein, it is difficult to make firm conclusions regarding the importance of different regions on the glycoprotein.

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Appendices

Appendix I: Solutions and reagents

Tissue culture media:

BHK Media

500ml BHK-21 GMEM

50ml heat inactivated foetal bovine serum (FBS) Australian origin

50ml Tryptose phosphate solution

5ml penicillin/streptomycin solution to 100U/ml penicillin and 100µl/ml streptomycin

HEK293-T 17 Media

500ml DMEM

50ml heat inactivated foetal bovine serum (FBS) Australian origin

15ml D (+) glucose solution to 4g/L

5ml penicillin/streptomycin solution to 100U/ml penicillin and 100µl/ml streptomycin

Bacterial cell culture media:

Luria –Bertaini (LB) Broth

10g tryptone

5g yeast extract

10g sodium chloride (NaCl)

950ml deionised water and autoclave to sterilise

Ampicillin or kanamycin to 100mg/ml

LB Agar

10g tryptone

5g yeast extract

10g NaCl

15g melted agar

Deionised water to 1l and autoclave to sterilise

Ampicillin or kanamycin to 100mg/ml

Molecular biological buffers:

TENS Buffer

10ml 1M Tris-HCL (pH 8.0)

2ml 0.5M EDTA

50ml 10% SDS

100ml 1M NaOH

Distilled water to 1l and autoclave to sterilise

Store at 37°C

TE Buffer (Tris-EDTA)

2ml 1M Tris-HCL (pH 8.0)

400µl 1mM EDTA

Sterile water to 200ml

TAE Buffer (Tris-acetate EDTA) (50X)

242g Tris base

57.1g glacial acetic acid

100ml 0.5M EDTA

Distilled water to 1l and autoclave to sterilise

Western blotting reagents:

12% separating gel

12 ml 30% Acrylamide (29:1)

11.25ml 1/1.5M Tris (pH 8.8)

300µl 10%SDS

6.5ml water

200µl 10% APS

50µl TEMED

Stacking gel

1.7ml 30% Acrylamide (29:1)

1.25ml 1M Tris (pH 6.8)

100µl 10%SDS

7ml water

100µl 10% APS

30µl TEMED

Transfer buffer

25mM Tris

190mM glycine

20% methanol

TBS-Tween 20

20mM Tris (pH 7.5)

150mM NaCl

0.2% Tween 20

Blocking buffer

100ml TBS-Tween 20

5% milk powder

Appendix II: List of Primers

A list of all primers used in this investigation is shown.

Name	Sequence	Melting Temperature (°C)
BBLV G for	GATCATGGTACCGCCACCATGCCAACTCAAGCCGTCCT	86.9
BBLV G rev	GATCATCTCGAGCTAAGCTTGACCTCCTGCAT	76.1
WCBV G PT For	GCGCGCGGTACCGCCACCATGGCTTCCTACTTTGCG	89.8
WCBV G PT Rev	GCGCGCCTCGAGTTATTGGGCAGTTTGTC	80.6
CVS G PT For	GCGCGCGGTACCGCCACCATGGTTCCTCAGGTTGTT	88.6
CVS G PT Rev	GCGCGCCTCGAGTTACAGTCTGATCTCACCTC	80.2
LBV D PT For	TATATATAGGTACCGCCACCATGAGTTACTCGATTTCAACACC	76.7
LBV D PT Rev	TATATATACTCGAGTTAGGCATTTGAGGAGCCCTTG	72.6
BBLV int G for	GAGACTGCAAAATGGTGC	59.4
BBLV int G rev	AAGTCATGAATGTTCACC	52.6
IKOV G for	TATATAGGTACCGCCACCATGGCTCAGTTGGTCACTTTGG	81.1
IKOV G rev	TATATACTCGAGCTAGAATGCAGAACTCTTGTAA	67
IKOV G int for	CGACCCTCAGGATGTGTTCCA	69.6
IKOV G int rev	AATTTAACTGTGTGTTTGTTC	53.3
LBV G (RV1) for	GCGCGCGGTACCGCCACCATGAGTCAACTAAATTTGATACTCT	84.6
LBV G (RV1) rev	GCGCATCCCGGGGCCACCATGAGTCAACTAAATTTGATACTCT	85.1
CVS G site swap checks for	ACGATACCAGACGAACCTGG	63.9
CVS G site swap checks rev	TAACTGAAGATTTCAACAACTCC	58.8
LBV G site swap checks for	AATTCCTGAAAAGATAGG	50.4
LBV G site swap checks rev	ACATCAACAAATTCATCGGCATCACC	72
pI.18 for	GGTGGAGGGCAGTGTAGTCT	63.2
pI.18 rev	GAAGACACGGGAGACTTAGT	57.4
CVS to LBV I rev	AGGCTTTCCACATAACGTGAGCC	69.1
LBV to CVS I rev	TAGTACCCCACATAATTTCAGTTTAC	60
LBV to CVS IV for	ACTTTCACATAGATGAAGTTG	53.3
LBV to CVS III for	TAAAAGCGTTCGCACTTGGAATGAAATAC	71.0

LBV to CVS III rev	ATTCCAAGTGCGAACGCTTTTATAATGAACG	73.4
CVS G Transfer into FL For	TATATATACCCGGGAAAGATGGTTCCTCAGGTTC	73.4
CVS G Transfer to FL Rev	TATATATAGCTAGCCAGTCCTTATCACAGTCTGATCTCACCTCC	74.8
LBV G Transfer into FL For	TATATATACCCGGGAAAGATGAGTCAACTAAATTTG	69.7
LBV G Transfer to FL Rev	TATATATAGCTAGCCAGTCCTTATCAGACATTAGAGG	67.6
CVS G transfer into FL HpaI For	TATATATAGTTAACAAAGATGGTTCCTCAGGTTC	65.5
LBV G transfer into FL HpaI For	TATATATAGTTAACAAGATGAGTCAACTAAATTTG	61.1
LBV G into FL PmeI for	TATATATATATATATAGTTTAAACAAGATGAGTCAACTAAATTTG	62.7
LBV G into FL XbaI rev	TATATATATATATATATCTAGACAGTCCTTATCAGACATTAGAGG	63.6
gib cSN rev	CTGTTTAAACGGTCTTTTGAGGGATGTTAATAG	69.3
gib LBV into cSN for	CATCCCTCAAAAGACCGTTTAAACAGATGAGTCAACTAAATTTGATACTC	77.8
gib LBV into cSN rev	TAGCCAGTCCTTACAGTCAGACATTAGAGGTACCC	73.0
gib cSN for	CTGTAAGGACTGGCTAGC	54.9
WCBV G transfer into FL HpaI For	TATATATAGTTAACAAGATGGCTTCCTACTTTGC	65.5
WCBV G transfer into FL NheI Rev	TATATATAGTCAGCACCTTGTTATTGGGCAGTTTGTC	71.8
IKOV G transfer into FL HpaI For	TATATATAGTTAACAAGATGGCTCAGTTGGTCAC	30.5
IKOV G transfer into FL NheI Rev	TATATATAGTCAGCAACCCACTAGAATGCAGAACTCTTG	30.7
CVSFSS to CVS IIa for	AGA GGGAAGAGAGCATCCAAAGG	67.7
CVSFSS to CVS IIa rev	CCTTTGGATGCTCTCTTCCCTCT	67.7
CVSFSS to CVS IIb for	TGAAGGATGTACCAACCTGTCCGAGTTCTCCTACATGGAAC	80.7
CVSFSS to CVS IIb rev	GTTCCATGTAGGAGAACTCGGACAGGTTGGTACATCCTTCA	80.7

Appendix III: Generic Scoring System for Mice Inoculated with Lyssaviruses

This scoring system was used to monitor and score all mice used in the experiments for this thesis. The scoring was conducted by members of both the Animal Services Unit and Wildlife Zoonoses and Vector Borne Disease Research Group at both daily assessments of the animals.

Clinical Score	Clinical Signs
0	No effect
1	Twitching in inoculated limb (in certain strains) Ruffled fur Hunched back Paralysis in inoculated leg
2	Spasms (in certain strains) Affected gait (slow or circular movements)
3	Hind quarter Paralysis Severe spasms (in certain strains)
4	Progressive paralysis
5	Prostration, permanent recumbency Death