

Investigation into the infectivity and neutralisation profiles of diverse lyssaviruses using a pseudotyped virus system

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Abstract

Rabies is an encephalitic disease with a mortality rate that approaches 100% in the absence of treatment. The World Health Organisation recognises rabies as a neglected tropical disease due to its disproportionate impact on poor and vulnerable populations in developing countries. Whilst vaccines exist to help prevent and control rabies, these are not always available or accessible to those at high risk. Rabies currently can only be treated effectively prior to the onset of symptoms and as immunoglobulin treatments are expensive and in short supply, and current vaccines require multiple doses to reach full efficacy, full courses of post-exposure prophylaxis may not be completed, leaving vulnerable individuals at risk.

Rabies is a zoonotic viral disease that is predominantly caused by the rabies virus. The rabies virus has a broad host range and is capable of infecting most mammalian species, though its main reservoir, and the source of most human infections, are canids, particularly dogs. Rabies can also be caused by non-rabies lyssaviruses, a group of viruses related to rabies virus that are mainly found in bats. Lyssaviruses have been isolated globally and whilst there are very few reports of rabies encephalitis caused by non-rabies lyssavirus species in humans, the general lack of awareness of lyssaviruses in bats can lead to delays in seeking treatment and increase the risks of developing rabies once exposed. Current vaccines are highly effective against rabies virus, but display less effectiveness against other lyssavirus species, with little to no protection provided against the more diverse lyssaviruses.

In this project, a lyssavirus pseudotype panel was generated using a range of lyssavirus species. This pseudotype panel was then used to investigate the neutralisation and tropism profiles of the lyssaviruses in question. In developing the pseudotype panel, it was identified that the choice of glycoprotein expression plasmid was vital for the generation of highly infectious pseudotype particles, and that the pI.18 plasmid was superior to pcDNA3.1.

A novel chimp adenovirus-based rabies vaccine was compared to a current human rabies vaccine for its capacity to elicit rabies-neutralising antibodies. A single dose of the novel vaccine was revealed to produce a comparable antibody response to that produced by the human vaccine following a full three-dose schedule.

Finally, the lyssavirus pseudotype panel was used to infect several animal cell lines to determine whether the viruses displayed any evidence of host adaptation. Some of the lyssavirus species showed increased infectivity in cells corresponding to their original host species, relative to a lab-adapted rabies virus strain.

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Abbreviations

-ev	*Ex vivo*
ΔEnv	Pseudotypes lacking an envelope glycoprotein
ABLV	Australian bat lyssavirus
ANOVA	Analysis of variance
APOBEC	Apolipoprotein B mRNA-editing catalytic polypeptide
Asn	Asparagine
bp	Base pair
bGH	Bovine growth hormone polyadenylation signal
BHK	Baby hamster kidney cells
BSL-2	Biosafety level 2
cDNA	Complementary DNA
ChAd	Chimp adenovirus
CMV	Cytomegalovirus
CRFK	Crandell Reese feline kidney cells
CVS	Challenge virus standard rabies virus strain
DesNi	*Desmodus rotundus* kidney cells
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EBLV	European bat lyssavirus
ERA	Evelyn-Rokitnicki-Abelseth rabies virus strain
FAVN	Fluorescent antibody viral neutralisation test
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
Flury-LEP	Flury-low egg passage rabies virus vaccine strain
G protein	Glycoprotein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HEK	Human embryonic kidney cells
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography

HPRT1	Hypoxine phosphoribosyltransferase 1
IC_{50}	Half maximal inhibitory concentration
IFN	Interferon
IKOV	Ikoma lyssavirus
kb	Kilobase
kDA	Kilodalton
KLD	Kinase, ligase and DpnI reaction
LB	Luria broth
LBV	Lagos bat lyssavirus
L protein	Large protein
mGluR2	Metabotropic glutamate receptor subtype 2
MLV	Murine leukaemia virus
MOKV	Mokola bat lyssavirus
M protein	Matrix protein
nAChR	Nicotinic acetylcholine receptor
NCAM	Neural cell adhesion molecule/CD56
NEAAs	Non-essential amino acids
NHP	Non-human primate
NKT	Asparagine, lysine, threonine
N protein	Nucleoprotein
p75NTR	p75 neurotrophin receptor
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEI	Polyethylenimine
PEP	Post-exposure prophylaxis
P protein	Phosphoprotein
PV	Pasteur virus rabies virus strain
RABV	Rabies virus
RFFIT	Rapid fluorescent focus inhibition test
RIG	Rabies immunoglobulin
RLU	Relative light units
RoNi	*Rousettus aegyptiacus* kidney cells
RNA	Ribonucleic acid

SAD	Street-Alabama-Dufferin rabies virus strain
SDM	Site-directed mutagenesis
Ser	Serine
SHIBV	Shimoni bat lyssavirus
STAT1	Signal transducer and activator of transcription 1
TAE	Tris –acetate-EDTA buffer
Thr	Threonine
TLR	Toll-like receptor
VSV	Vesicular stomatitis virus
WCBV	West Caucasian bat lyssavirus
WHO	The World Health Organisation

Chapter 1 - Introduction

1.1 Introduction to rabies and lyssaviruses

Rabies is a disease that has plagued humanity for centuries and there are hints of its presence in texts from ancient Mesopotamia [1]. With the near inevitability of death upon the development of rabies symptoms and lack of treatment options, vaccination is the primary tool available for protection against lethal infection.

Rabies is caused by the classical rabies virus (RABV), a member of the *Rhabdoviridae* family and the archetypal species of the *Lyssavirus* genus. Whilst originally it was believed that RABV was the sole cause of rabies it is now known that other lyssaviruses are capable of causing disease, with symptoms that are clinically indistinguishable from those caused by RABV [2].

1.1.1. Lyssavirus transmission and epidemiology

Rabies is a zoonotic disease and is transmitted through bites from infected animals, with dogs (*Canis lupus familiaris*) being the cause of 99% of all human rabies cases [3]. RABV is, however, capable of infecting many different animal species and has multiple host reservoirs, making complete eradication unlikely [4].

The principal reservoir of RABV in Europe is the red fox (*Vulpes vulpes*), with raccoon dogs (*Nyctereutes procyonoides*) also becoming an important reservoir following their importation into Western Russia in the 1920s [5]. In Africa, domestic dogs are the major reservoir for RABV, though there are frequent spill-over infections into other carnivorous species [6]. RABV is mainly found in raccoons, skunks and insectivorous bats in North America [5], whilst in Latin America, haematophagous and insectivorous bats are the major reservoirs for RABV [7].

Whilst RABV is common in bats in America, RABV has yet to be isolated in bats outside of America although other lyssaviruses have been predominantly

isolated from old world bats. In contrast, no lyssaviruses other than RABV have been identified in the Americas [8]. The reason for this is currently unknown, though lyssaviruses appear to be evolutionary more ancient than RABV, so potentially this could be due to some sort of founder effect, with RABV in America originating in the colonial period [8].

Another hypothesis is that lyssaviruses may have originated within bats and an ancient RABV strain may have circulated in bats in the ancient super-continent of Gondwana. When the South American and African continents split apart, RABV continued to evolve within bats in the Americas and underwent a host-shift to terrestrial carnivores in Africa. This hypothesis does not explain why RABV has never been isolated from bats in Africa, though there is the possibility that it is present and yet to be discovered due to limited surveillance [4,9].

The evolutionary history of lyssaviruses is not well understood and molecular clock based methods that are commonly used to extrapolate evolutionary timescales may not be appropriate for use with viruses that evolve under strong purifying selection such as lyssaviruses [4].

The majority of lyssaviruses have been found in bats, with the exception of Mokola virus (MOKV) and Ikoma virus (IKOV). Whilst MOKV was originally isolated in 1968, its reservoir species is currently unknown. MOKV has been isolated from a variety of mammals including shrews (*Crocidura spp.*) [10] and cats (*Felis catus*) [11]. IKOV has so far only been isolated once, from an African civet (*Civettictis civetta*) [12], though due to the solitary nature of this host it is believed to have been an incidental infection rather than the reservoir host for IKOV [13].

Other lyssaviruses, such as European bat lyssavirus 1 (EBLV-1) and Australian bat lyssavirus (ABLV), have been predominantly found in bats but have been known to cause spill-over infections in livestock such as sheep and horses [14,15].

Whilst lyssaviruses primarily infect mammals, there is some evidence to suggest that they can infect other types of animals as well. RABV has been shown to infect a wide range of cell types *in vitro* including mosquito cells and fish cells [16] and there has even been a naturally occurring case of rabies identified in a chicken (*Gallus gallus domesticus*) [17]. Likewise, MOKV has also been shown to be capable of replicating in insect cells [18]. Whilst all lyssaviruses seem to have a broad host tropism, there may be differences in tropism between them. For example, it has been shown that the CHO-K1 (Chinese hamster ovary) cell line is resistant to infection by ABLV [19] but is permissive to infection by RABV [20].

Rabies is a zoonotic disease and is transmitted through bites from infected animals, with dogs being the cause of 99% of all human rabies cases [3]. The incubation period for rabies is variable, with symptoms generally appearing anywhere from 2 weeks to 2 years after initial exposure [21], though there are some reports of cases where the initial exposure was believed to have taken place more than 4 years prior to the onset of symptoms [22,23]. The incubation period appears to be somewhat affected by the number and location of bites, with shorter incubation periods being observed for cases with multiple bites and for bites to the head and neck regions [21]. The variable incubation rate can make it difficult to trace the initial exposure, particularly in the case of bat-transmitted rabies as bat bites and scratches may not leave noticeable lesions [24].

One of the more unusual ways of transmitting rabies is through organ transplantation. There have been multiple reports of rabies transmission through organ transplantation where the original infection was not diagnosed and as multiple organs can be harvested and transplanted into different patients this can lead to multiple patients succumbing to rabies infection [25–27]. With the recipients of organ donations being kept on immunosuppressive drugs, patients may fail to develop an antibody response to the infection, increasing the difficulty of diagnosis [25].

The World Health Organisation (WHO) classifies rabies as a neglected tropical disease and the majority of cases occur in Africa and Asia [3]. It is estimated that there are approximately 60,000 human deaths caused globally by rabies each year and up to 50% of those receiving post-exposure prophylaxis (PEP) are children under the age of 16 [28,29].

The economic costs associated with rabies infection are mainly due to the productivity losses caused by premature death, but are also affected by the cost of PEP and loss of livestock [30,31]. Whilst the loss of livestock affected by rabies only accounts for 6% of the global economic burden, this cost is disproportionately borne by subsistence farmers in rural areas [29]. The predicted burden of rabies is likely to be an underestimate due to limited surveillance and lack of reporting in developing countries [28].

Some countries previously considered to be rabies-free, such as Australia and the United Kingdom (UK), have been found to be host to bats carrying lyssaviruses. ABLV was identified in fruit bats (*Pteropus alecto*) in Australia in 1996 [32] and since then has gone on to cause fatal infections in two horses [15] and three humans [33–35]. In the UK, *Myotis daubentonii* bats have been found to be infected with European bat lyssavirus 2 (EBLV-2) [36] and this virus has caused a single fatal case in a human in Scotland [37]. EBLV-1 has been found in bats across Europe and has previously caused two confirmed human infections [38]. EBLV-1 was identified in serotine bats (*Eptesicus serotinus*) in the UK for the first time in 2018 [39]. These cases illustrate the risks posed by lyssaviruses even in developed countries, due to lack of awareness.

1.1.2. Clinical rabies symptoms

Whilst rabies is typically considered lethal once symptoms arise, the likelihood of developing rabies following an untreated bite from a rabid animal can vary from 37% to 80% depending upon wound severity and location [40].

Rabies patients may experience prodromal symptoms prior to developing full rabies symptoms. This can include pain or itching at the site of the bite wound and these symptoms may spread to involve the whole limb [40].

Rabies infections can present in one of two clinical forms: encephalitic or paralytic. The most common presentation of rabies is the encephalitic (or "furious") form, which accounts for 80% of rabies infections [41]. The encephalitic form of rabies is characterised by agitation and excitement interspersed with periods of lucidity. Hydrophobia, hypersalivation and throat spasms, leading to difficulty swallowing, are also associated with this form of rabies. [42]

The paralytic form of rabies can be difficult to diagnose as it differs from the traditional rabies symptoms and may be mistaken for other neurological conditions such as Guillain-Barré syndrome [40]. Paralytic rabies is characterised by muscle weakness and flaccid paralysis in the early stages before progressing to coma and death [42].

1.1.3. Treatments and vaccines for rabies

It is possible to treat rabies prior to the onset of symptoms by providing a patient with PEP following exposure to a rabid animal. PEP is very effective in preventing rabies and there are very few reports of PEP failure leading to patient death. Most reports of PEP failure are due to deviation from the recommended protocol, though there are some rare cases where patients have succumbed to rabies despite being provided appropriate treatment following exposure [43]. PEP consists of wound care, a course of rabies vaccinations and administration of rabies immunoglobulin (RIG) [3].

The first rabies vaccine was developed by Louis Pasteur in 1885 by isolating RABV from a rabid cow [44], passaging the virus in rabbits and drying the nerve tissue to attenuate the virus [45]. Modifications were made to Pasteur's vaccine by Claudio Fermi and David Semple through the addition of phenol to inactivate the virus and to act as a preservative, enabling long term storage [46]. Nerve

tissue vaccines are no longer recommended by the WHO due to their adverse effects.

Current vaccines are produced in cell culture or embryonated eggs, using human diploid cells, Vero cells, purified chick embryo cells or embryonated duck eggs. These vaccines use attenuated strains of RABV in order to provide protection. One downside of these vaccines is that multiple doses are required to protect against rabies and this affects both the cost and availability of vaccination [3].

New vaccines are currently being developed in an attempt to address some of the shortfalls of current rabies vaccines such as cost and immunogenicity. Novel vaccines have high hurdles to face in that they need to be at least as effective as current vaccines and should elicit a consistent immune response across different human populations [47]. Novel methods of administering rabies vaccines such as through oral formulations or biodegradable needles would also be valuable and may help to reduce costs, but are not currently being explored for rabies [47].

Novel rabies vaccine candidates that are currently being investigated include protein vaccines, genetic vaccines, genetically modified rabies vaccines, adjuvanted vaccines and viral vector vaccines.

Protein vaccines involve the administration of recombinant viral protein; in the case of rabies this is the glycoprotein. The rabies glycoprotein is capable of eliciting full, humoral protection against rabies when it is properly folded and glycosylated, however it has to be in its trimeric form to induce neutralising antibodies [48]. Whilst protein vaccines are safe to use, they are not very immunogenic and require the use of an adjuvant. This, along with the requirement for careful purification, means a protein-based vaccine will likely not be cost-effective [49].

Genetic vaccines include both DNA and RNA vaccines and involve the delivery of a gene or transcript. They are similar to protein vaccines in that they only include the relevant subunit, however as the antigenic protein is produced within the host this ensures that the protein is folded correctly and contains the appropriate modifications [47]. An mRNA-based vaccine for rabies has been tested in humans and proved to be safe with mainly mild adverse effects, however neutralising antibodies were only generated when administered via a needle-free device. The immune response also faded after a year, indicating that it would not be suitable as a pre-exposure vaccine [50].

Current human rabies vaccines use laboratory adapted, attenuated rabies virus strains which have been inactivated using reagents such as formalin or β-propiolactone [51,52]. Live attenuated viruses are capable of replicated inside the body and stimulate an immune response similar to that resulting from a natural infection. Inactivated virus vaccines are incapable of replicating within the body, are less immunogenic and require multiple vaccine doses to stimulate an immune response which is predominantly humoral, with little cell-based immunity being developed [53]. Genetically modified virus vaccines seek to produce attenuated rabies virus strains through modifying the viral genes. Rabies virus strains modified to lack the matrix gene were shown to be both replication deficient and to elicit high titres of neutralising antibodies [54]. Another option for genetic modification is the development of rabies virus strains expressing two glycoprotein genes as the increased expression of the glycoprotein may make inactivated viruses more immunogenic [55].

Adjuvants may be used with inactivated vaccines to help boost the immune response, enabling fewer doses to be required to provide protection. Most human rabies vaccines do not contain adjuvants or use aluminium salts as an adjuvant, a common adjuvant that has been used in vaccines since the 1920s [56,57]. Aluminium salts promote Th-2 immune responses in mice and are known to be poor stimulators of T-cell responses in humans [58]. As aluminium salts lack potency, new adjuvants are being developed as a replacement [56]. PIKA (a synthetic analogue of dsRNA and a TLR3 agonist) and CV8102 (an RNA-

based TLR7/8 agonist) are two potential adjuvants that have been studied for the use alongside current rabies vaccines and both led to improved antibody responses [59,60].

Viral vector vaccines involve the modification of a virus vector to express a foreign antigen from the virus of interest. Various vectors have been studied for their use as rabies vaccines, particularly vaccinia vectors [61] and adenovirus vectors [62]. Viral vectors are safe and can be highly immunogenic, however there is an issue regarding the immune response to the vector. As such, vectors with a low seroprevalence are sought after for use as vaccine vectors [63].

Vaccines are also available for the control of rabies in wildlife populations. These vaccines are delivered orally and are enclosed within an edible bait. In Europe, wildlife vaccines use attenuated live viruses derived from the Street-Alabama-Dufferin (SAD) RABV strain [64], whilst the USA and Canada use recombinant vaccinia or adenovirus vectors expressing the glycoprotein of the SAD or Evelyn Rokitnicki Abelseth (ERA) RABV strains [62,65].

Post-exposure prophylaxis involves the administration of anti-rabies immunoglobulin (RIG) alongside vaccination to inhibit the spread of infection [66]. There are two types of RIG that have typically been used alongside vaccination, human RIG and equine RIG. Both of these treatments are often in short supply and can be costly, leading many in developing countries to be treated by vaccination alone [67]. As such, effective monoclonal antibodies (mAbs) are being investigated for use as a replacement to RIG.

When developing mAbs for use as a viral treatment there are several factors that need to be taken into consideration, the main one being the risk of viral escape. As viruses are known for their high mutation rates, the use of a single mAb to treat an infection would provide a strong selective pressure and likely lead to proliferation of viruses with mutations in the targeted region and treatment failure. As such, multiple antibodies that target different sites on the viral glycoprotein should be used in combination as this means that multiple

mutations would be required for the virus to escape neutralisation [68]. Any mAbs that are intended to be used in the treatment of rabies will also have to show that they are capable of neutralising a diverse range of RABV strains in order for there to be confidence in their use as a comprehensive treatment [68].

Several mAbs have been developed for treatment of RABV infections are at varying stages of the testing process.

Rabishield is the only mAb that has completed phase 3 clinical trials and is licensed for use in India. It contains a single human IgG1 mAb, Rab1, that binds a conformational epitope on the RABV glycoprotein. As it contains just a single mAb there are some concerns that Rabishield will not be able to effectively neutralise all RABV strains [69], but it provides a valuable treatment option where RIG or ERIG are unavailable [70,71].

Rabimabs is made up from two murine mAbs, M777-16-3 and 62-71-3, which bind to separate sites on the RABV glycoprotein. Rabimabs has completed phase 1 and 2 clinical trials and is currently progressing through phase 3 trials [71].

SYN023 is a cocktail of two humanised mAbs, CTB011 and CTB012, that has completed phase 1 and 2 clinical trials, with phase 3 currently being planned. The two mAbs bind to separate sites on the RABV glycoprotein, with CTB011 binding to antigenic site III and CTB012 binding to a region of discontinuous conserved residues that do not form part of a known antigenic site [72].

The antibody cocktail CL184 contains 2 mAbs, CR57 and CR4098, which target distinct, non-overlapping epitopes on the RABV glycoprotein. CL184 has completed two phase 1 studies performed in the US and India which found it to be well tolerated [66]. CL184 has also completed three phase 2 studies however no results have been published and CL184 has been withdrawn from clinical development [71].

Researchers are also regularly searching for new mAbs that could have the potential to form the basis of a rabies mAb treatment, such as the broadly neutralising mAb RVC58 [69].

Antibodies and vaccines directed against RABV may provide some cross-reactivity and protection against lyssavirus species in phylogenetic group 1 [44] but have little to no effect on viruses from the other phylogenetic groups [73]. The true impact of lyssaviruses on public health is unknown as whilst there are some known cases of these viruses infecting humans [74], these viruses cause diseases that are clinically indistinguishable from classical rabies and additional testing to determine the causative viral agent is not always carried out [75]. With the current push to eliminate canine-mediated rabies by 2030 [76,77], it is possible that this could lead to an ecological niche that other lyssaviruses are capable of exploiting, particularly because most of these viruses are found in bats which are both difficult to immunise and protected species [78].

A small handful of individuals have survived rabies without vaccination or PEP. The most well-known of these cases was a patient who was treated with an aggressive strategy now known as the Milwaukee protocol, that involved placing her into a therapeutic coma by administering high doses of intravenous sedatives in order to protect the brain from the effects of autonomic dysregulation [79,80]. The patient was also treated with ketamine and amantadine, N-methyl-D-aspartate (NMDA) receptor antagonists, to protect against glutamate-mediated excitotoxicity in the brain [80]. The antiviral drug ribavirin was also provided in the hope that it would help to provide some protection against RABV despite the drug showing limited penetration into the central nervous system [79,81]. Ribavirin is a purine analogue that acts as an antiviral by leading to the equal incorporation of cytidine and uridine into viral RNA, resulting in hyper-mutation [82]. Whilst ribavirin has been shown to be effective against RABV *in vitro* [83], studies in mice [83] and human case studies [84] have shown a lack of efficacy.

Despite many adjustments being made to this protocol, the Milwaukee protocol has failed to live up to initial hopes and evidence suggests it may be ineffective [80]. Both ketamine and amantadine have limited evidence to suggest they display anti-rabies efficacy [85,86] and have led to disappointing results in human case studies [80]. It is unknown why the original patient treated with this protocol survived, however she was already expressing RABV-specific antibodies prior to the start of treatment [79], therefore it is possible that she was only able to recover due to her strong immune response. The patient was initially exposed to rabies after receiving a bite from a bat, therefore it is also possible that her recovery was due to bat variants of RABV being less virulent than canine variants.

A survey of populations in the Peruvian Amazon identified a small handful of individuals that were expressing neutralising anti-RABV antibodies in their serum, only one of which reported prior RABV vaccination. This could imply that exposure to bat-transmitted rabies is not necessarily fatal, supporting the theory that bat variants of RABV are less virulent [87], however there have been no further studies to confirm this.

Rabies mortality following exposure to a rabid animal varies from 38-80% in the absence of treatment and is affected by the location and severity of the wound sustained, with traumatic bites to the head and neck region having an increased likelihood of developing rabies symptoms and have a reduced incubation period [40]. As the number of viral particles transmitted from exposure to a rabid bat will be much fewer than that from a canine exposure, the odds of achieving a productive infection and the subsequent development of rabies symptoms may be reduced. This may potentially lead to an increased incubation time as well, allowing for the body to mount an immune response prior to the onset of symptoms and increasing the chances of recovery. The risk of developing rabies from exposure to a rabid bat is, however, still present despite the fact that wounds sustained are smaller and more superficial in nature. This may potentially be because bat RABV variants are capable of replicating within the epidermis, allowing the virus to amplify prior to entering

the nervous system in order to compensate for the lower viral load administered [88].

1.2 Lyssavirus structure

1.2.1. Lyssavirus classification

Lyssaviruses can be split into approximately three phylogroups based upon their genetic sequences and antibody neutralisation patterns [89–91]. Lyssaviruses were originally assigned to serotypes based upon their serological cross-reactivity [92], however as molecular biology techniques have advanced they have been separated into genotypes (and more recently species) based upon their genetic variation [93].

Members of the different phylogroups do not cluster by geographical location, with representatives of all three phylogroups found throughout Europe, Africa and Asia [13].

Where multiple isolates of a single lyssavirus have been sequenced, they often appear to cluster geographically due to genetic drift caused by a lack of proof-reading [94–96]. Evolutionary analyses of lyssaviruses have shown that they are subject to purifying selection, which is potentially due to them infecting an immune-privileged site leading to a reduction in selective pressure [97,98].

1.2.2. Genome organisation

Lyssaviruses are negative-sense single-stranded RNA viruses with a genome of around 12kb in size which codes for five proteins: the nucleoprotein (N protein), the phosphoprotein (P protein), the matrix protein (M protein), the glycoprotein (G protein) and the RNA-dependent RNA-polymerase (L protein) [41].

The order of genes in the RABV genome helps to determine the abundance of viral mRNAs as transcription occurs in a stop-start manner ignoring inter-genic regions. As transcription doesn't always restart after stopping, reduced amounts

of mRNA are produced the further down the genome they are situated [99]. This leads to large amounts of structural proteins (nucleoprotein etc.) being produced and comparatively lower amounts of enzymatic proteins (polymerase) (Fig 1.1). This enables the production of optimal amounts of viral proteins at low enough concentrations to prevent cell cytotoxicity and the activation of immune responses [41,100].

Once large amounts of N and P proteins have been produced, mRNA is encapsidated, leading to full length mRNA replication, rather than transcription [101]. Virus particles then exit host cells by budding, enveloping themselves in host cell membranes which have virus glycoproteins embedded into them [41].

The nucleoprotein is a phosphorylated protein approximately 450 amino acids in size that interacts with the phosphoprotein in order to encapsidate the RNA genome [102,103]. This helps to protect the RNA genome from ribonuclease activity and triggers the transition from RNA transcription to replication [101,104]. The nucleoprotein may also play a role in immune avoidance by preventing RIG-I recognition of RABV genomic RNA [105].

The phosphoprotein interacts with the nucleoprotein and helps to confer specificity of encapsidation of genomic RNA by the nucleoprotein [106]. The phosphoprotein also acts as a co-factor for the polymerase in order to aid in viral replication [107]. As the phosphoprotein is capable of interacting with

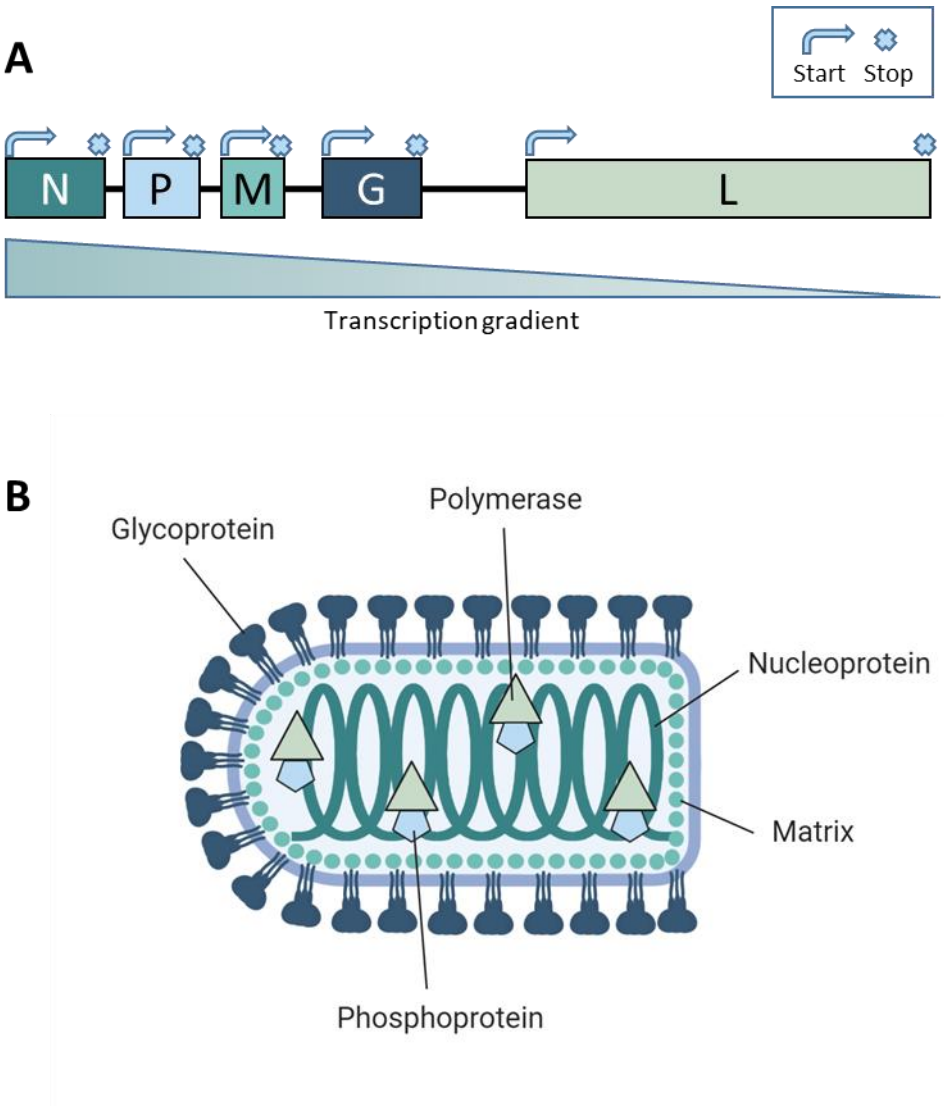


Figure 1.1 The genome organisation of lyssaviruses
Schematic showing the lyssavirus genome organisation (A) and the location of the lyssavirus proteins within the virion (B).

STAT1 and inhibiting IFN pathways, it is also considered to play an important role in evading the immune response [108].

The matrix protein, at approximately 202 amino acids long, is the smallest of the lyssavirus proteins [104]. The matrix protein is involved in condensing the ribonucleoprotein complex and interacting with the cytoplasmic tail of the glycoprotein, giving it an important role in viral assembly and budding [109]. When RABV is mutated to lack the M protein, the subsequent virions lack the typical bullet shape and instead form long, rod shaped structures [109].

The glycoprotein is the most well-studied lyssavirus protein [104] and is found embedded into the host-derived lipid envelope. The glycoprotein coats the surface of the virus with the exception of the blunt end of the bullet [110]. Due to its location on the surface of the virion, the glycoprotein plays an important role in receptor binding and antibody recognition [111].

The L protein is the largest lyssavirus protein and encodes the RNA-dependent RNA polymerase, playing an important role in viral transcription and replication [112]. The L protein likely catalyses all enzymatic reactions involved in RNA synthesis and processing similar to the Vesicular stomatitis virus (VSV) L protein, and has previously been shown to play a role in catalysing mRNA capping [113].

It is rare for negative-sense RNA viruses to undergo recombination and this is also true for lyssaviruses, however there is some evidence to suggest that there may have been some recombination within the polymerase gene for some strains of rabies virus [114]. Recombination requires multiple strains of a virus to be infecting a host at the same time. As rabies has a relatively long incubation period (two weeks to two years) co-infection is a feasible possibility [21].

1.2.3. The lyssavirus glycoprotein

The lyssavirus glycoprotein is a 65 kDa protein [115] of approximately 524 amino acids in length, dependent on viral species. It is made up of four domains:

the signal peptide, the ectodomain, the transmembrane domain and the cytoplasmic domain (Fig 1.2).

The signal peptide is 19 amino acid residues in length and is a hydrophobic domain [116] that is involved in directing glycoprotein insertion into the membrane of the endoplasmic reticulum, where the signal sequence is subsequently cleaved from the mature protein [117]. As such, amino acid numbering for identifying sites in the mature protein traditionally starts from the 20th residue [118].

The ectodomain is the largest domain of the lyssavirus glycoprotein at 439 amino acid residues long [104] and protrudes from the viral membrane. The ectodomain is the antigenic portion of the lyssavirus glycoprotein and is responsible for both receptor binding and antibody escape. No crystal structure has yet been resolved for the lyssavirus glycoprotein although it has been predicted using computer modelling [119]. However another Rhabdovirus, vesicular stomatitis virus (VSV), glycoprotein has had its crystal structure resolved in both its pre-fusion and acidic conformations [120,121] (Fig 1.3). The VSV glycoprotein structure has therefore been used in the past to visualise lyssavirus antigenic sites [122]. Whilst the sequence identity between the lyssavirus and VSV glycoproteins is quite low at ~23% [122], they are structurally homologous [119] and 5/7 disulphide bonds in the ectodomain are conserved between VSV and lyssaviruses [123]. There are 14 cysteine residues within the ectodomain of the lyssavirus glycoprotein and these are conserved across all lyssavirus species, with EBLV-1 having an additional 15th cysteine residue at position 206, directly beside the conserved cysteine at position 207.

The transmembrane domain is a 21 amino acid hydrophobic domain of the glycoprotein and is involved in anchoring the glycoprotein to the viral membrane. The remaining ~42 amino acids make up the cytoplasmic tail. The cytoplasmic tail of the glycoprotein interacts with the matrix protein, which coats the ribonucleoprotein complex and is required for virus particle assembly [109].

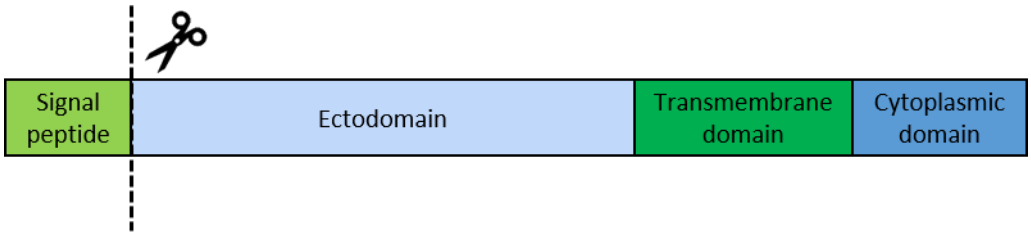


Figure 1.2 Domains of the lyssavirus glycoprotein
Schematic depicting the domains of the lyssavirus glycoprotein.

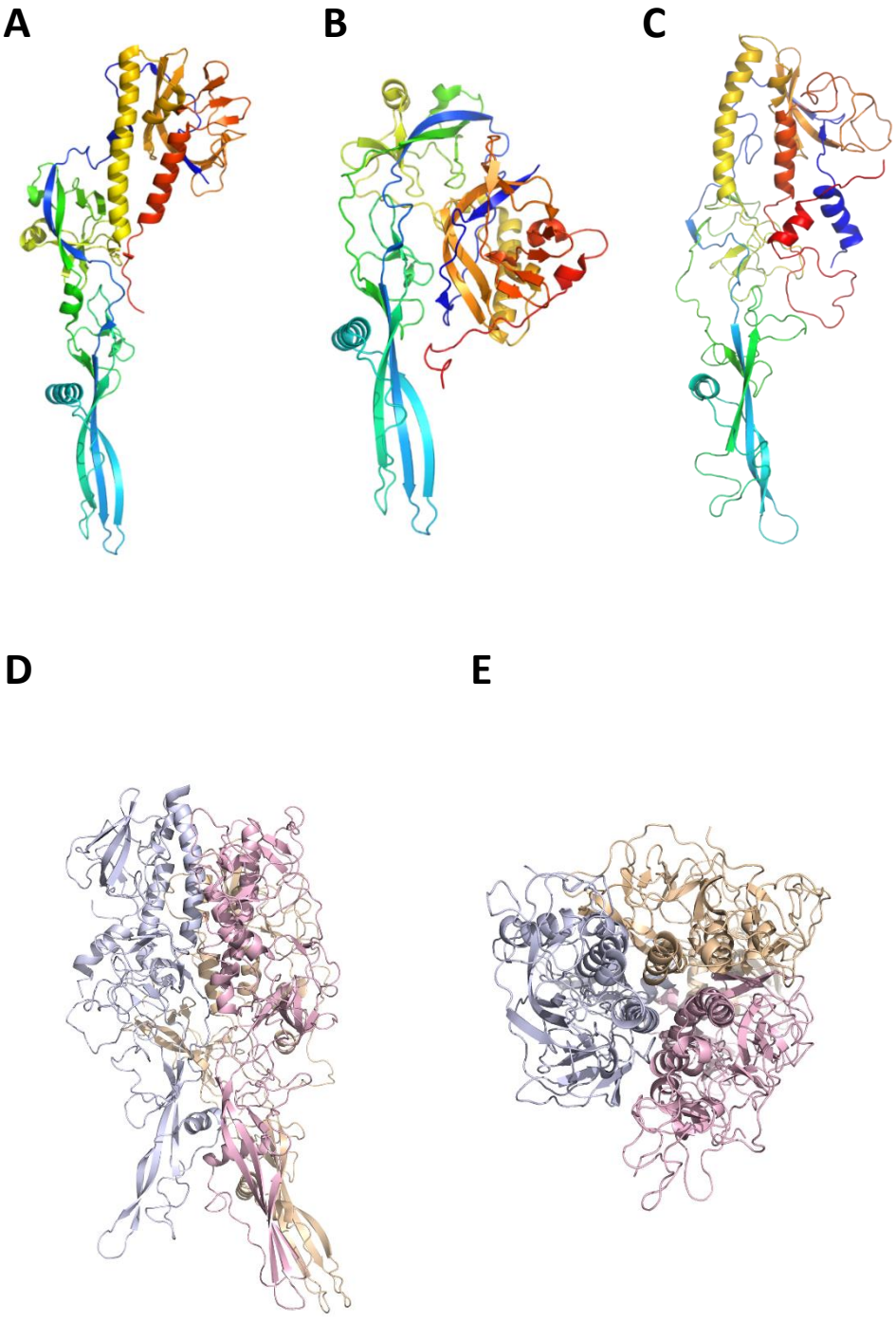

A
B
C
D
E

Figure 1.3 Protein structures of the VSV and RABV glycoproteins
Protein structures of the VSV glycoprotein in the A) acidic conformation (PDB: 5I2M [121]) and B) pre-fusion form (PDB: 5I2S [120]). The RABV glycoprotein pre-fusion model (PMDB: PM0079619 [119]) is also included as C) a monomer, D) a trimer viewed from the side and E) a trimer viewed from the top, with individual monomers coloured accordingly.

The lyssavirus glycoprotein forms trimers on the surface of the viral particle and these have been visualised using electron microscopy [124]. Lyssaviruses enter host cells via clathrin-coated pits [125] and the viral membrane fuses with the vesicular membrane in order to release the viral genome into the cells [41].

The structure of the glycoprotein is sensitive to pH and the glycoprotein undergoes conformational changes as the pH is decreased and, unusually for viral fusion proteins, these changes are reversible [126]. Both VSV and lyssavirus glycoproteins are examples of Type III fusion proteins, which share structural similarities with both Type I and Type II fusion proteins [127]. The fusion domain of the VSV glycoprotein is located at the tip of a β-pleated sheet and has a similar structure to the fusion domain of a Type II fusion protein. The structure of the VSV glycoprotein is also similar to a Type I fusion protein in that it contains a central α-helical coiled-coil and forms a six-helix bundle in its post-fusion form. Unlike Types I and II fusion proteins however, the fusion loops of the VSV glycoprotein are not protected by subunit interfaces and are instead exposed on the surface of the structure [127].

N-linked glycosylation is a common post-translational modification which involves the attachment of oligosaccharides to the amide nitrogen atom of the asparagine residue within the motif Asn-X-Ser/Thr, where X is any amino acid except proline [128]. Glycosylation can help to enable protein folding and transport to the cell membrane [129]. In the case of viral glycoproteins, glycosylation can also help to mask antigenic sites, aiding the virus in evasion of antibody detection [130].

The lyssavirus glycoprotein, as indicated by the name, is glycosylated and the number of glycosylation sites varies by species. RABV strains typically have two glycosylation sites within the ectodomain, at positions 37 and 319, though site 37 is poorly glycosylated [129]. This is potentially due to the amino acid at the X position of this site being a leucine, which is associated with inefficient glycosylation [131] or because this site contains serine rather than threonine at the third position, which also leads to less efficient glycosylation [132]. It has

previously been shown that RABV can escape a neutralising monoclonal antibody targeting this region via the mutation S39T, which enables this site to be glycosylated more efficiently, allowing the virus to evade antibody binding [133].

The glycosylation site at position 319 is efficiently glycosylated [129] and is conserved across all lyssavirus species. This site also aligns with a glycosylation site in the VSV glycoprotein at position 320 [134], which may indicate that this glycosylation site has an important role in glycoprotein structure or function. It has previously been shown that glycosylation of this site is required for the secretion of soluble rabies glycoprotein [135].

Some RABV strains contain a third glycosylation site within the ectodomain, particularly lab-adapted virus strains such as the ERA strain which has a third glycosylation site at position 247 [129] or the challenge virus standard 11 (CVS-11) strain which has a third site at position 204 [136]. The glycosylation site at position 204 is glycosylated in some strains of CVS-11 but not others and may be related to the presence of a glutamic acid residue at position 198 though further research is required [136]. These additional glycosylation sites are not commonly found in wild-type RABV strains so they may be viral adaptations to improve infectivity in cell culture.

Other lyssaviruses in phylogroup 1 contain only a single glycosylation site within the ectodomain, the conserved site at position 319. Group 2 and 3 lyssaviruses are more heavily glycosylated, with the number of glycosylation sites within the ectodomain varying from 2 to 5. All group 2 and 3 lyssaviruses contain a conserved glycosylation site at position 202 in addition to the site at 319. The role of these glycosylation sites and their importance for pathogenesis is still currently unknown.

Viral glycoproteins are heavily targeted by the immune system as they are exposed on the virus particle's exterior surface. Antigenic sites on the glycoprotein are regions that are recognised by multiple antibodies and are

easily accessible [137]. The lyssavirus antigenic sites were originally identified by generating virus escape mutants in the presence of neutralising monoclonal antibodies, then characterising the viral mutants based on their neutralisation profiles and mutations within the glycoprotein sequence [138].

Lyssaviruses have four major antigenic sites (sites I, II, III and IV) and a single minor site (site a), most of which are conformational and can only be recognised based on the glycoprotein's folded structure [137]. The majority of the antigenic sites are short, continuous stretches of amino acids ranging from 2-8 amino acids in length. The exception to this is antigenic site II, which is discontinuous and split into two discrete sites, with site IIa located at amino acids 198-200 and site IIb at 34-42 [139].

Antigenic sites I and III do not overlap and so antibodies targeting each site can be used in conjunction. The same is true of antigenic sites I and II. Antibodies targeting sites II and III however compete with each other for binding, indicating that they are located in close proximity to one another [118,138] (Fig 1.4 and 1.5).

Antigenic site-swap experiments between RABV and the group 2 lyssavirus Lagos bat virus (LBV) have indicated that antigenic site II may have particular importance in neutralising phylogroup 2 lyssaviruses [140].

Interestingly, a single amino acid change from arginine at position 333, which is located within antigenic site III, has been previously linked with RABV spread and pathogenicity [141]. Whilst other mutations within antigenic site III can affect neutralisation, only position 333 is linked to pathogenesis [142].

1.3 Lyssavirus entry receptors

Most information relating to lyssavirus receptors is based on RABV as this has been extensively researched due to its neurotropism making it an attractive research tool [143]. Whilst there is still a lack of clarity around RABV and lyssavirus entry receptors, several potential candidates have been proposed.

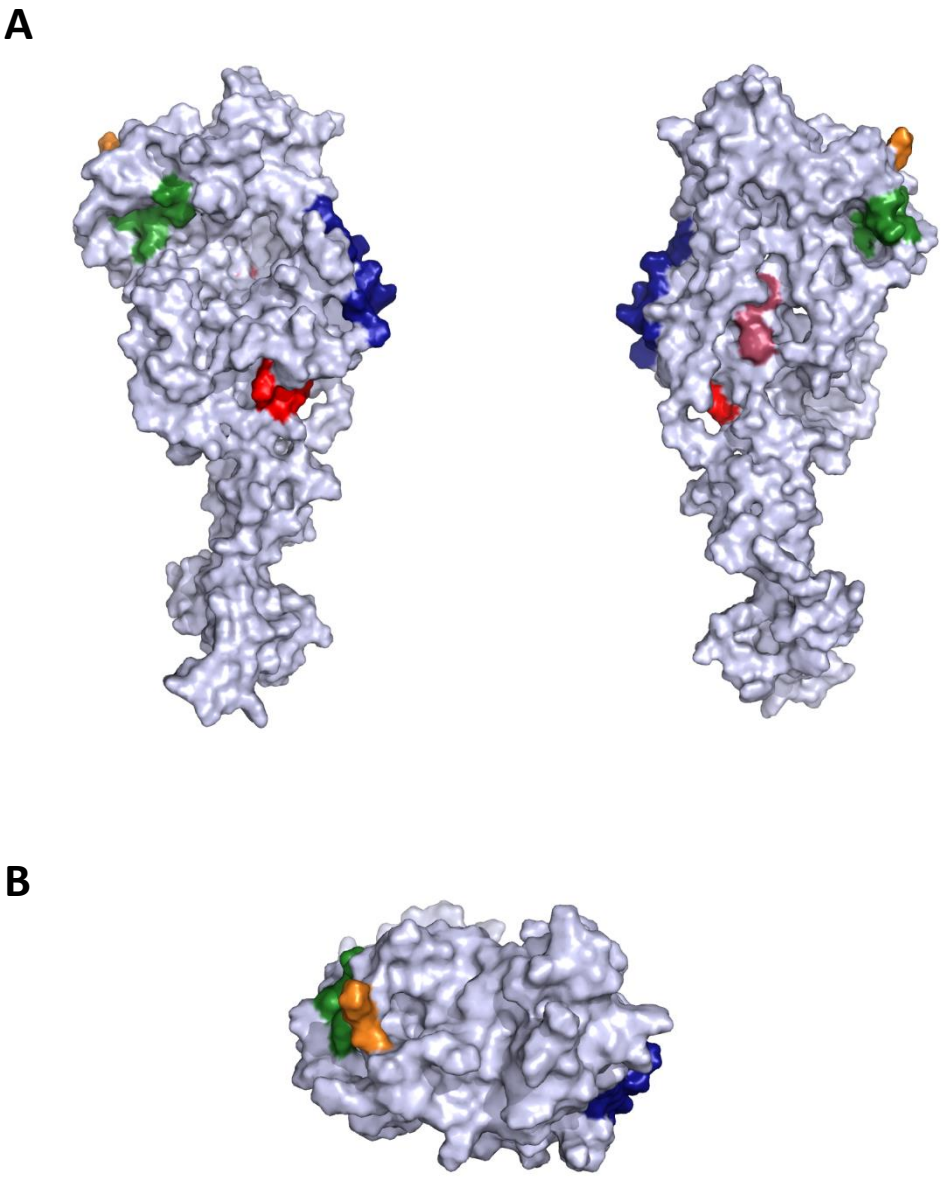


Figure 1.4 Location of antigenic sites on the RABV glycoprotein monomer
RABV antigenic sites displayed on the surface of the RABV glycoprotein monomer model (PMDB: PM0079619 [119]) viewed from the sides (A) and the top (B). Antigenic site I: red, II: blue, III: green, IV: pink, minor site a: orange.

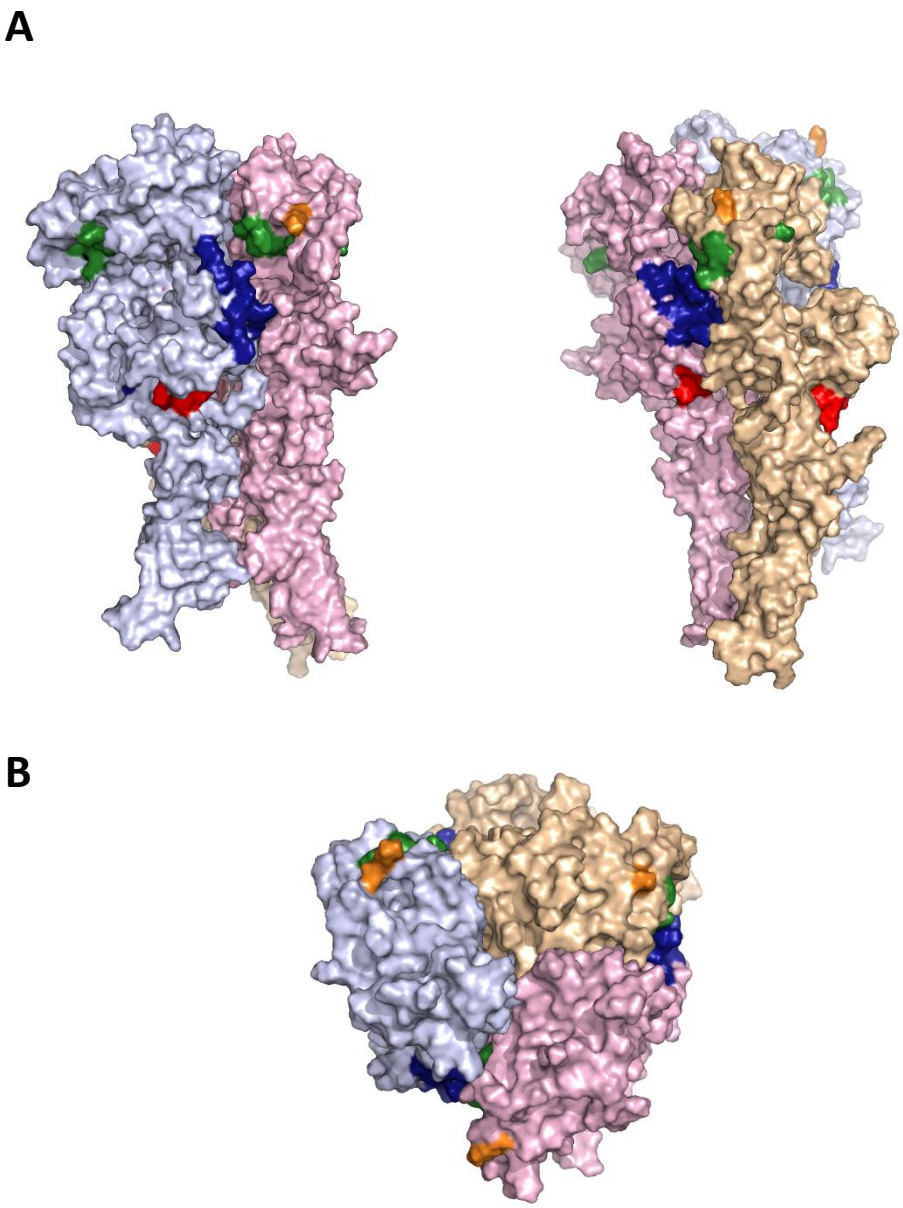


Figure 1.5 Location of antigenic sites on the RABV glycoprotein trimer
RABV antigenic sites displayed on the surface of the RABV glycoprotein trimer model (PMDB: PM0079619 [119]) viewed from the sides (A) and the top (B). The co-localisation of antigenic sites II and III can be seen at the interface of two monomers in section A. Antigenic site I: red, II: blue, III: green, IV: pink, minor site a: orange. Individual glycoprotein monomers have been coloured accordingly.

1.3.1. Nicotinic acetylcholine receptor

One of the first potential receptors identified for RABV was the nicotinic acetylcholine receptor (nAChR), when it was observed that the RABV antigen could be detected in neuromuscular junctions with a similar distribution to nAChRs. It was also seen that treatment of myotubes with ligands of the nAChR such as α-bungarotoxin and tubocurarine led to a reduction in RABV infection [144].

nAChRs are ligand-gated ion channels that can be found throughout the nervous system and are present in the neuromuscular junction. When the neurotransmitter acetylcholine binds to a nAChR, an ion channel is opened leading to a change in the electrical state of the cell [145]. nAChRs have complex pentameric structures that are assembled from diverse subunits [146] with different subunit combinations associated with different locations [147]. nAChRs found on muscle cells may have two different subunit compositions: α1, β1, δ and γ or α1, β1, δ and ϵ, with each version containing two copies of the α1 subunit [145]. Neuronal nAChRs can be homopentamers or heteropentamers and contain various combinations of the subunits α2, α3, α4, α5, α6, α7, α9, α10, β2, β3 and β4. RABV has been reported to bind to the α1 subunit of the neuromuscular nAChR [148].

Supporting the role of nAChRs as a RABV receptor is the fact that the RABV glycoprotein has amino acid sequence homology with snake venom neurotoxins which are capable of binding nAChRs, with the greatest similarities being seen in conserved regions where the toxin binds to the nAChR [149]. Anti-idiotype antibodies raised against RABV-neutralising antibodies have been found to be capable of binding nAChR, providing further evidence to support binding of RABV to nAChR [150].

The α1-nAChR is located on muscle cells at the post-synaptic membrane, and therefore cannot be used by RABV for entry into neurons [151]. It has been suggested that binding to the nAChR helps to concentrate the presence of rabies virus particles in the synapse, making it easier for them to then enter the cells

[144]. Another possibility is that RABV may use the nAChR to enter muscle cells where it may undergo preliminary rounds of replication prior to entering the nervous system [41]. Recent data has shown that RABV may also be capable of binding the α4 subunit of nAChR, which is most commonly found in the central nervous system (CNS), however little is known about this interaction [152].

Rabies viruses are also capable of infecting cells that don't express nAChRs, indicating that this is not the only receptor the virus is able to use to enter cells [153].

1.3.2. Neural cell adhesion molecule

The neural cell adhesion molecule (NCAM) is another potential RABV receptor which can be found on both the pre- and the post-synaptic membrane in neuromuscular junctions [154] and plays a role in synapse formation and maturation [155].

NCAM is a neuronal surface glycoprotein that, as its name suggests, binds to other proteins to aid in cellular adhesion and migration [156]. NCAM is a member of the immunoglobulin superfamily and contains five immunoglobulin-like domains and two type III fibronectin-like domains [20]. There are at least 27 different NCAM mRNA splice variants, giving rise to multiple NCAM isoforms [156]. The three major NCAM isoforms have identical ectodomains, with the main differences being in the transmembrane and cytoplasmic tail regions, with NCAM-140 and NCAM-180 having cytoplasmic tails of differing lengths and NCAM-120 lacking a cytoplasmic tail and instead linking to the plasma membrane via a phosphatidylinositol-glycan transmembrane anchor [20].

NCAM was originally identified as a potential receptor when it was observed that NCAM was present on cell lines susceptible to RABV infection, but absent on resistant cell lines, with expression levels broadly correlating to RABV susceptibility [20]. When resistant cells were transfected with the *NCAM1* gene they became susceptible to RABV [20,157]. It was also shown that cells treated

with antibodies directed against NCAM or with the NCAM ligand heparan sulphate, they showed a reduction in cell susceptibility to RABV [20].

NCAM is unlikely to be the sole receptor for rabies virus however, as knockout mice still succumbed to rabies virus infection, albeit with a delayed response compared to wild type mice [20].

1.3.3. P75 neurotrophin receptor

The third potential RABV receptor identified is the p75 neurotrophin receptor ($P75^{NTR}$). This receptor is mainly expressed during development or following injury or neurodegeneration [151]. The $p75^{NTR}$ does not contain any catalytic domains and instead partners with different neurotrophin and non-neurotrophin receptors that allow it to influence a variety of different cellular pathways [158]. The $p75^{NTR}$ does not appear to be found in the neuromuscular junction [159]. It was identified as a potential RABV receptor after a randomly primed cDNA library was used in an attempt to identify RABV-binding molecules [160].

Whilst it has been shown that the RABV glycoprotein is capable of binding to mammalian $p75^{NTR}$ [161] this does not necessarily translate into a role as a RABV entry receptor; indeed some neurons which express $p75^{NTR}$ are unable to bind to RABV [162]. There is also no *in vivo* evidence that $p75^{NTR}$ is a RABV receptor. As this receptor is also not found in the neuromuscular junction it is therefore unlikely have a role in RABV entry to the CNS [162].

1.3.4. Metabotropic glutamate receptor subtype 2

A new candidate receptor for RABV has been identified recently in the metabotropic glutamate receptor subtype 2 (mGluR2) [163]. mGluR2 is a G-protein coupled receptor that is expressed on the pre-synaptic axon terminal [164]. RNA interference was used in HEK293 cells to screen for potential RABV receptor candidates, with downregulation of mGluR2 being associated with RABV replication inhibition. Antibodies targeted to mGluR2 were also capable of

blocking RABV infection *in vitro* [163]. Further research is required in order to elucidate the role this receptor may play in lyssavirus entry.

1.3.5. Non-traditional receptors

As well as traditional receptors, there is also some evidence that RABV can bind to gangliosides. Gangliosides are glycolipids that are found on eukaryotic cell membranes, particularly in nervous tissue. When cells are treated with neuraminidases, which cleave sialic acid residues, the cells gain resistance to RABV infection, whilst the addition of crude gangliosides restores RABV infectivity. Purified gangliosides were capable of restoring some cell susceptibility to RABV, but not completely as with crude preparations. This could indicate that gangliosides work as part of a multi-component complex in order to act as a receptor for RABV [165].

Heparan sulphate is a glycosaminoglycan that is abundant on cellular surfaces. Treatment of cells with heparan sulphate was previously shown to block RABV entry and it was assumed that this was due to heparan sulphate binding to NCAM competing with RABV for binding [20]. Recent research has shown that heparan sulphate is capable of binding to RABV itself and cellular expression led to increased RABV adhesion to cells, though due to its ubiquitous nature heparan sulphate likely only acts in aiding attachment, rather than as an entry receptor [166].

Lyssavirus entry into cells is a complicated issue that is far from being solved and it is likely that multiple receptors are involved in cellular entry.

1.4 Viral pseudotyping

Viral pseudotyping is a method that is available for the study of highly pathogenic viruses in low level containment facilities. Pseudotyped viruses are replication-incompetent viruses that express foreign viral envelope proteins.

Retroviruses and lentiviruses are capable of incorporating other cellular proteins, including envelope proteins, from other viruses, allowing their tropism to be altered [167]. The retroviral or lentiviral vector backbones have

been modified through the removal of genes to render them replication-incompetent and thus non-pathogenic [168].

Gammaretroviral vectors, such as mouse leukaemia virus (MLV), and lentiviral vectors, such as human immunodeficiency virus 1 (HIV-1), are the most commonly used pseudotype cores, however VSV-based vectors are also available, though are more technically challenging to create [169,170].

VSV is capable of producing pseudotypes as VSV virions are able to freely incorporate a wide variety of membrane proteins into their envelopes and are capable of budding even in the absence of VSV-glycoprotein [171]. When cells are co-infected with VSV and a second virus, VSV-based pseudotypes can be formed. VSV pseudotypes are created by initially transfecting cells with a Δenv-reporter plasmid and four support plasmids which contain the remaining VSV genes. A T7 promotor source is required and the virus is also complemented with VSV-g. The virus is then propagated and used to infect cells that are expressing a heterologous glycoprotein [171]. The VSV-based pseudotypes need to be assessed for their neutralisation profile as the VSV glycoprotein is efficiently pseudotyped so pseudotypes may contain a mixture of glycoproteins. As an extra infection step is required in the production of VSV-based pseudotypes, they are more technically challenging to produce that retroviral or lentiviral pseudotypes, which can be produced in a single transfection reaction [170].

Retroviruses and lentiviruses are also capable of incorporating glycoproteins from other viruses into their envelopes and so can be used as pseudotypes. Retroviral and lentiviral pseudotypes are typically created using a three-plasmid system, though two- and four-plasmid systems are also available [170]. Separate plasmids are used to increase the flexibility of the system, by allowing the switching of envelope glycoprotein plasmids to easily generate different viral pseudotypes. Separate plasmids also helps to reduce the risk of recombination as multiple recombination events would need to occur to produce replication-competent virions. This is particularly important when

producing HIV-env pseudotypes on an HIV-1 backbone as these have been known to give rise to replication-competent viruses [172].

Various genes have been removed from HIV-1 vectors in order to improve safety. The accessory genes *Vif, Vpr, Vpu* and *Nef* are required for natural viral infections but are not necessary in pseudotype systems.

Vif is a late stage protein that acts during virus budding to prevent the host cell factor apolipoprotein B mRNA-editing catalytic polypeptide (APOBEC) from entering the budding particle and leading to the accumulation of dC to dU mutations in the viral genome [173]. Vif is only required however when infecting non-permissive cell types such as T-cells and macrophages, as would occur in a natural HIV infection. As pseudotypes are typically produced in 293-T cells, Vif is not required [174].

The Vpr protein aids in the nuclear transport of the HIV-1 pre-integration complex and is required for viral replication. The matrix protein and integrase protein are also capable of promoting nuclear entry and can compensate for a lack of Vpr at a high multiplicity of infection (MOI) [175]. As Vpr seems to mostly be important when infecting terminally differentiated macrophages, it is therefore not required for the production of effective pseudotypes, however a lack of Vpr may be detrimental when using HIV-1 vector-based gene delivery to macrophages [174].

Vpu has two major roles in HIV-1 infection: CD4 downregulation and promoting virus release from cells. Whilst CD4 plays a major role in HIV-1 entry into cells, its high affinity for the HIV-Env protein can impact on viral assembly. Vpu leads to a reduction in CD4 expression by targeting CD4 for degradation through ubiquitination [176]. Vpu also helps to promote virus release by downregulating Tetherin, a host-cell protein that binds HIV-1 particles to the surface of the cells, preventing their release [176]. As CD4 and Tetherin are not expressed on permissive HEK-293T cells, effective pseudotypes can still be produced in the absence of Vpu [174].

The accessory protein Nef has multiple functions and is capable of interacting with several host cell factors. Nef is known to improve virus infectivity where fusion at a neutral pH is possible, and has no effect where endosome acidification is required for fusion to take place [177,178]. Nef is therefore not required for the creation of many pseudotypes, though may need to be provided for optimal expression of others, particularly retroviral glycoproteins [179].

The *Tat* gene has also been removed and replaced with a strong constitutive promotor in many three plasmid pseudotype systems [169].

The three plasmids involved in the creation of pseudotypes are the envelope plasmid, which contains the glycoprotein gene of the virus of interest; the *gag/pol* plasmid, which contains the structural proteins that make up the core of the virus particle and the replicative proteins responsible for integrating the reporter gene into the host genome; and the reporter plasmid, which contains the reporter gene flanked by long terminal repeats and also containing a packaging signal to ensure its RNA is incorporated into the virus particle [169]. As the packaging signal is only present with the reporter gene, this is the only genetic material present in the pseudotyped virus particle, preventing further viral replication [169]. In a four-plasmid system, the *Rev* gene has been removed from the *gag/pol* plasmid and moved onto a separate plasmid to improve safety. There are also pseudotype systems where the reporter gene is encoded on the *gag/pol* plasmid, so only two plasmids are required [170].

Vector choice can affect pseudotype titre, with HIV-1 vectors typically leading to higher levels of reporter gene expression than MLV vectors for certain glycoproteins such as the CVS-11 RABV strain [180] and VSV [181]. It is known that lentiviruses are capable of transducing all cells within a population, whilst MLV is only capable of transducing dividing cells, which may lead to a reduction in reporter gene expression [170]. Some hepatitis C pseudotypes, on the other hand, have higher levels of reporter gene expression when pseudotyped with an

MLV backbone than a HIV-1 backbone [182], indicating that the most appropriate backbone is glycoprotein dependent.

Pseudotyped viruses have many advantages for viral research particularly when investigating viruses that would require high containment levels thanks to their good safety levels. By switching the envelope plasmid it is also possible to create a wide variety of virus pseudotypes relatively quickly and more simply than having to propagate live virus strains. As retroviruses and lentiviruses integrate their genes into the host-cell chromosomes they are capable of inducing long-lasting gene expression, so can also be used for purposes such as gene therapy.

Pseudotyping is a flexible system that has a variety of uses and pseudotyped viruses have been used to target specific cells for treatment [183], provide evidence of host adaptation [184], investigate how protein expression levels affect infectivity [185] and to explore the mechanisms of antibody neutralisation [186].

Lyssavirus pseudotypes have also been developed and used to identify broadly-acting neutralising antibodies [69], the relative importance of antigenic sites [140] and to assess the seroprevalence of lyssaviruses in bats [187].

There are downsides to pseudotyped viruses however, particularly that they are a model of infection and results obtained using them may not necessarily be representative of what would happen in a natural infection. It is known that pseudotyped viruses are typically more sensitive to neutralisation *in vitro* than live viruses are *in vivo* [188]. This may be differences in glycoprotein expression levels between pseudotypes and live viruses which can affect the accessibility of the glycoprotein to neutralising antibodies. Despite these drawbacks, pseudotyped viruses are still a useful research tool and data produced using them can provide a direction for future avenues of research using live viruses and animal models.

In summary, pseudotypes are a flexible tool that are particularly useful in the investigation of highly pathogenic viruses that would otherwise require high containment facilities to research.

1.5 Project outline

As there are still many unanswered questions surrounding lyssavirus entry and there is a requirement for improved rabies vaccines, this project set out to investigate the cross-neutralisation of lyssavirus strains by vaccinated serum, including investigating a novel adenovirus-based vaccine, and lyssavirus host-tropism through the use of viral pseudotypes.

To this end, a lyssavirus pseudotype panel was established and optimised prior to being used to examine the neutralising capacity of vaccinated sera against various lyssavirus species. The lyssavirus pseudotype panel was also used in infectivity assays against cells from different animal species to determine whether the lyssaviruses displayed any evidence of host adaptation.

Chapter 2 – Materials and methods

2.1 Lyssavirus glycoprotein selection

The RABV-medoid glycoprotein was kindly provided by ReiThera SRL, Italy. Lyssavirus isolates were selected from all three phylogroups to be used for pseudotype studies. Eight of these lyssavirus glycoproteins (Rabies virus CVS-11 (RABV-CVS) (EU352767) [189], European bat lyssavirus 1 (EBLV-1) (EU352768) [190], European bat lyssavirus 2 (EBLV-2) (EU352769) [191], Lagos bat virus (LBV) (HM623779) [192], Mokola virus (MOKV) (HM623780) [193], Shimoni bat virus (SHIBV-ev) (GU170201) [194], West Caucasian bat virus (WCBV) (EF614258) [195] and Ikoma virus (IKOV-ev) (JX193798) [196]) were kindly provided in the pI.18 plasmid by Dr Edward Wright, University of Sussex [180,197]. Of these lyssaviruses, only the SHIBV and IKOV glycoprotein sequences were derived *ex vivo*, whilst the other strains had been passaged at least once prior to sequencing.

Genbank was also searched in order to identify additional lyssavirus glycoproteins that were sequenced directly *ex vivo*, and an additional four lyssaviruses were selected (Rabies virus (RABV-GrD-ev) (LM645022) [198], Rabies virus (RABV-GtB-ev) (KF656698), Australian bat virus (ABLV-ev) (AF426304) [98] and Lagos bat virus (LBV-ev) (EU259198) [199]) and synthesised by Geneart Gene Synthesis (Thermo Fisher). VSV glycoprotein was used as a control.

2.2 Plasmids

Lyssavirus glycoproteins provided by Dr Edward Wright were present in the pI.18 expression plasmid. pcDNA3.1 was also used as a glycoprotein expression plasmid. The phCMV5349 plasmid contained the MLV-*gag/pol* gene and the pTG126 plasmid contained the firefly luciferase reporter gene. The pNL4-3.luc.R-E- (pNL4-3) plasmid is a 1st generation pseudotype plasmid containing the HIV-1 proviral genome with a frameshift mutation in the *env* and *vpr* genes and with luciferase replacing the *nef* gene. psPAX2 is a 2nd generation pseudotype plasmid that expresses HIV-1 *gag, pol, rev* and *tat* with the pCS-Luc

transfer plasmid encoding the reporter gene luciferase. The VSV glycoprotein was originally in the pMD2.G plasmid and later cloned into the pI.18 glycoprotein expression plasmid.

2.2.1. Plasmid preparation

Plasmids were transformed into chemically competent *E. coli* (Stellar competent cells, Takara Clontech) via heat shock. Bacterial cells were removed from -80°C storage and thawed on ice. Up to 5 ng plasmid DNA was added to 50 µl cells and incubated on ice for 30 mins. The cells were then heat shocked at 42°C for 45 secs then placed on ice for another 5 mins recovery. 250 µl SOC medium (Takara Clontech) was added to the cells and they were incubated for 1 hr at 37°C in a shaking incubator before being spread onto LB-ampicillin plates and left to grow at 37°C overnight.

Single colonies were picked from LB-ampicillin plates and added to 5 ml LB broth containing 100 µg/ml working concentration of ampicillin. Cultures were incubated at 37°C overnight in a shaking incubator. Plasmids were extracted from the bacteria using the GenElute plasmid mini-prep kit (Sigma-Aldrich). Briefly, up to 5 ml of bacterial culture was centrifuged at >12,000xg in a fixed angle centrifuge. The cell pellet was lysed in an alkaline lysis solution, then neutralised. Debris was pelleted and the supernatant added to a previously prepared binding column. The column was washed twice and centrifuged to dry, with the plasmid being eluted in 50 – 100 µl elution solution in a fresh tube.

Plasmid concentrations were quantified using a Nanodrop spectrophotometer and plasmids were stored at 4°C.

Glycerol stocks of bacteria containing plasmids were prepared by taking 500 µl of transformed bacteria that had been cultured overnight in LB-ampicillin broth and adding 3 drops of 100% glycerol from a Pasteur pipette, to produce a final concentration of approximately 12% glycerol. Cultures were vortexed to homogenise the solution and stored at -80°C.

Additional plasmid preparations were produced from the glycerol stocks by scraping the frozen glycerol with a pipette tip and adding to 50 ml LB-Ampicillin broth and incubating at 37°C overnight in a shaking incubator. Plasmids were extracted using the GenElute plasmid midi-prep kit (Sigma-Aldrich). Briefly, 50 ml of bacterial culture was centrifuged at 5,000xg for 10 mins in a fixed angle centrifuge. The cell pellet was lysed in an alkaline lysis solution then neutralised. Debris was removed by filtering through a filter syringe on a column placed on a vacuum manifold. The column was washed twice and left on the vacuum for 10 mins to dry. The column was then removed and placed into a collection tube and the plasmid was eluted in 1 ml Elution solution.

2.2.2. Plasmid digestion

The pI.18 and pcDNA3.1 plasmids used in InFusion cloning were linearised with EcoRI. A 50 µl reaction was set up containing 5 µl EcoRI (100 U) restriction enzyme (New England Biolabs), 5 µl CutSmart buffer (New England Biolabs), approximately 10 µg plasmid, and nuclease-free water to bring the reaction volume to 50 µl. The reaction mixture was incubated at 37°C overnight then heat inactivated at 65°C for 20 mins. The digested plasmid was then purified using the Nucleospin gel and PCR clean up kit (Machery-Nagel). Briefly, the plasmid was mixed with the 2 volumes of buffer and placed into a binding column. The column was then washed twice and centrifuged to dry. The plasmid was then eluted in 15 – 30 µl buffer NE and quantified using the Nanodrop spectrophotometer.

2.3 Cloning

Lyssavirus glycoproteins were cloned into the pcDNA3.1 plasmid through TOPO directional cloning (Invitrogen) and InFusion cloning (Takara Clontech) and into the pI.18 plasmid through InFusion cloning.

2.3.1. TOPO directional cloning

PCR primers were designed to amplify the whole glycoprotein sequence and included a CACC tag on the 5' end of the forward primer (Fig 2.1). The glycoprotein sequence was amplified by PCR. The reaction mix contained 2.5 µl

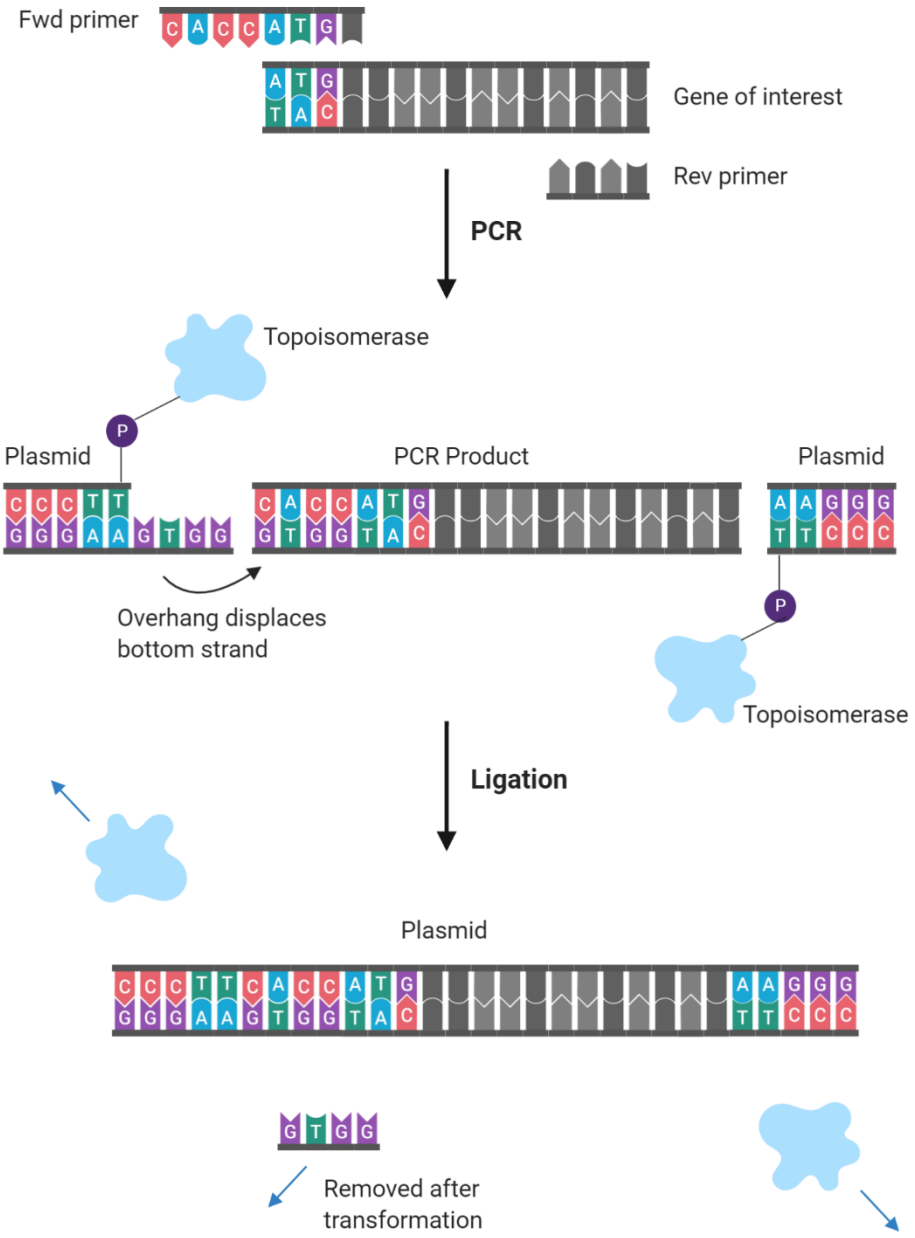


Figure 2.1 Overview of TOPO directional cloning
Schematic to show the process of TOPO directional cloning. The CACC bases in the forward primer are complementary to those present in the overhang on the cloning vector, enabling directional cloning. When the PCR product is ligated into the plasmid, the phospho-tyrosyl bond between the DNA and the topoisomerase is broken, releasing the topoisomerase.

5x Q5 reaction buffer (New England Biolabs), 0.62 µl forward and reverse primers (6.2 pmol), 0.25 µl dNTPs (2.5 mM, Sigma Aldrich), 0.5 µl DNA template, 0.125 µl Q5 Hot Start High-Fidelity DNA polymerase (0.25 U, New England Biolabs) and 7.875 µl nuclease-free water. The PCR reaction was carried out using the following cycling conditions:

Initial denaturation: 98°C for 30 secs
25 cycles
    98°C for 30 secs
    Primer-specific annealing temperature for 20 secs
    72°C for 30 secs per kb
Final extension: 72°C for 2 mins

PCR products were then run on an agarose gel to confirm amplification. The PCR product was then diluted 1 in 5 and a TOPO reaction mix produced containing 0.5 µl TOPO vector (pcDNA3.1, Invitrogen), 0.5 µl salt solution (1.2 M NaCl, 0.06 M $MgCl_2$, Invitrogen), 0.5 µl diluted PCR product and 1 µl sterile water. The reaction mix was incubated for 30 mins at room temperature then 1.25 µl was added to 50 µl competent *E. coli* and transformation continued as detailed in section 2.2.1. Cloning success was verified through colony PCR using T7 forward and bGH reverse primers (Appendix I) targeting regions of the plasmid on either side of the cloning site. PCR products with bands of the appropriate size were sent for Sanger sequencing for confirmation.

2.3.2. InFusion cloning

Primers were designed to amplify the entire glycoprotein sequence and included a CACC Kozak sequence at the 5' end of the forward primer and 15 bp overlaps that are complementary to the ends of the linearised plasmid at the 5' ends of both the forward and reverse primers (Fig 2.2). These overlaps ensured the glycoprotein sequences were inserted in the correct orientation The glycoprotein was amplified through PCR using the same protocol as the TOPO directional cloning. PCR products were run on an agarose gel to confirm amplification. The remaining PCR product was purified using the Nucleospin gel

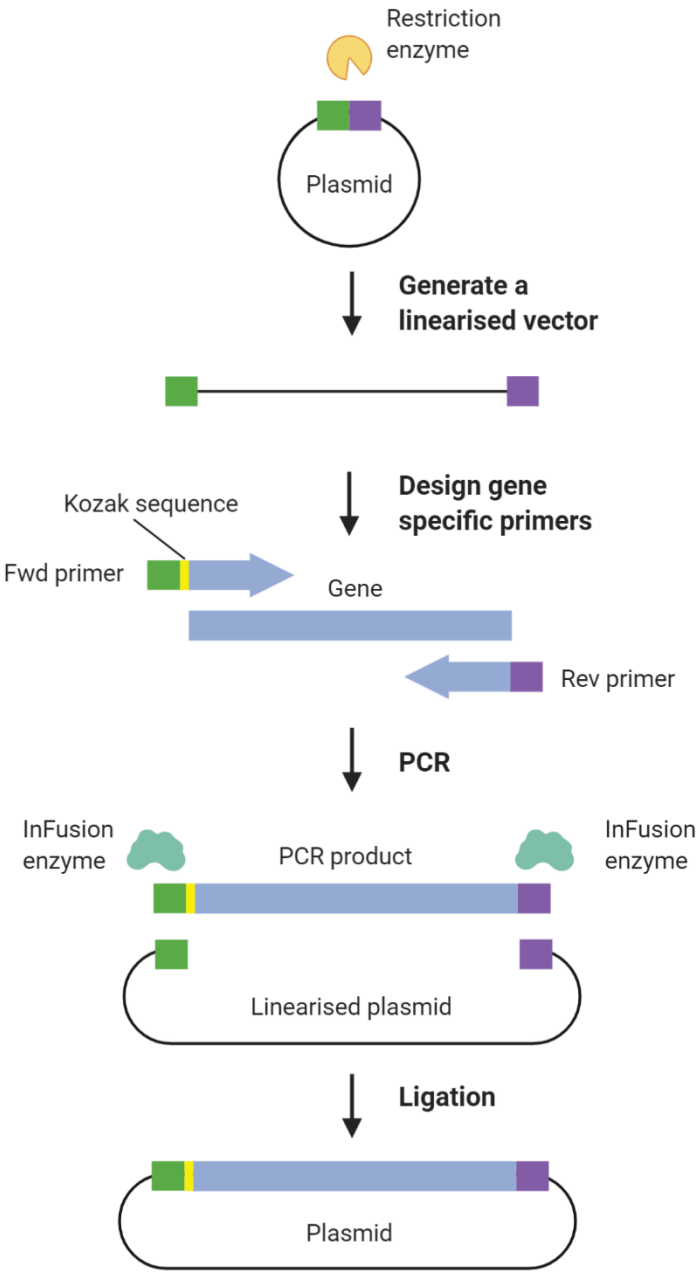


Figure 2.2 Overview of InFusion cloning
A schematic to show the process of InFusion cloning. The PCR product requires 15bp regions at both the 5' and 3' end that are homologous to the ends of the linearised vector. The InFusion enzyme recognises these regions of homology and joins the fragment to the vector in the correct orientation.

and PCR clean up kit (Machery-Nagel), following the same protocol as the plasmid purification in section 2.2.2. The InFusion reaction mix was then produced containing 1 µl purified PCR product (50 – 100 ng), 1 µl linearised plasmid vector (50 – 100 ng) and 0.5 µl 5x InFusion HD enzyme pre-mix (Takara Clontech). The reaction mix was then incubated at 50°C for 15 mins then 2.5 µl was transformed into competent *E. coli* as described in section 2.2.1.

2.4 Colony PCR

Colony PCR was performed on transformed bacterial colonies to confirm the plasmids contained the correct insert. The PCR reaction mix contained 1.5 µl 10x buffer (Qiagen), 0.6 µl forward and reverse primer (6 pmol), 0.6 µl dNTPs (2.5 mM), 0.075 µl HotStar *Taq* polymerase (0.4 U, Qiagen) and 11.6 µl nuclease-free water. Individual colonies were then selected from transformed bacterial LB-ampicillin plates and added to the PCR reaction mix. Colonies were then stabbed into a fresh LB-ampicillin plate for easy identification once their identity had been confirmed. The PCR reaction was carried out using the following cycling conditions:

Initial denaturation: 95°C for 15 mins
25 cycles
    95°C 20 secs
    Primer-specific annealing temperature for 20 secs
    72°C for 30 secs per kb
Final extension 72°C for 2 mins

2.4.1. Agarose gel electrophoresis

2% agarose gels were made by heating 2 g agarose powder (Sigma Aldrich) in 100 ml 1x TAE buffer (Sigma Aldrich). 2.5 µl ethidium bromide (Sigma Aldrich) was added to the gel once it had cooled, but before it solidified. 2.5 µl 10x FastDigest Green Buffer (Thermo Scientific) was added to the PCR product to act as a loading dye and 5 µl of PCR product-loading dye mix was loaded onto the gel. 2.5 µl GeneRuler DNA ladder mix (Thermo Scientific) was used as a DNA marker. The gel was run in 1x TAE containing 5 µl ethidium bromide at 90 V for

36 mins. Bands were visualised with UV light. PCR products of the appropriate size were sequenced by Source Biosciences to confirm the sequence was correct.

2.5 Site-directed mutagenesis

Site-directed mutagenesis was performed on the pI.18 plasmid to prepare it for use in InFusion cloning, to add a cysteine residue to the RABV-GtB-ev glycoprotein sequence and to add a glycosylation site to several phylogroup 1 lyssavirus species. Primers were designed with 5' ends annealing back-to-back, with the appropriate mutation contained towards the 5' end of the primers.

For the SDM to prepare the pI.18 plasmid for InFusion cloning, primers were designed flanking the EBLV-1 glycoprotein in pI.18, with the forward primer starting at the 3' end of the glycoprotein and the reverse primer starting at the 5' end of the glycoprotein. These primers contained the pcDNA3.1 homologous sequence and new EcoRI restriction site at their 5' ends. The forward primer also included a single bp change to remove the EcoRI site that was already present immediately downstream of the glycoprotein to ensure there would only be one restriction site in the final empty plasmid vector.

SDM was able to efficiently remove the glycoprotein present in the pI.18 vector and to edit the EcoRI site that was present, however the insertion of the new EcoRI site flanked by pcDNA3.1 homologous sequence caused a major issue. When the colonies were sequenced there were 1-8 bps missing from the sequence, corresponding to the 5' ends of the SDM primers. These missing bases were right in the middle of the new EcoRI restriction site and so would prevent the plasmid from being digested.

The primers were re-synthesised using HPLC purification to ensure all the primers were of the correct length and would not be missing any bases from the 5' ends, however this had no effect. The primers were then re-designed so that two bases of the insertion were moved from the reverse primer to the forward primer, so that when re-ligation occurred, the join would be in a different place

(Appendix I). This approach was successful, with 3/4 colonies containing the full insertion.

PCR was carried out using the Q5 PCR reaction mix as detailed in section 2.3.1. PCR products were visualised via agarose gel electrophoresis. The remaining PCR product was used in the kinase, ligase DpnI (KLD) reaction. The reaction mix contained 0.5 µl PCR product, 2.5 µl KLD reaction buffer (New England Biolabs), 0.5 µl KLD enzyme mix (New England Biolabs) and 1.5 µl nuclease-free water. The reaction mix was incubated at room temperature for 5 mins then transformed into competent *E. coli* as described in section 2.2.1. The resulting colonies were screened by PCR and sequenced for confirmation.

2.6 Cell lines

HEK-293T, BHK-21 and CRFK cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% heat inactivated foetal bovine serum (FBS, Gibco) and 1% non-essential amino acids (NEAAs, Gibco). DesNi vampire bat cells were maintained in DMEM with 10% FBS, 1% NEAAs and with the addition of 1% penicillin/streptomycin (Gibco). RoNi/7.1 bat cells were maintained in DMEM plus 10% FBS, 1% NEAAs, 1% penicillin/streptomycin and were additionally supplemented with 1% sodium pyruvate (Gibco).

2.6.1. General cell culture techniques

Cells were passaged twice a week until they reached a maximum passage number of 50, at which point they were discarded in Distel-Trigene. To passage the cells, spent medium was poured off and the cells were briefly washed in phosphate-buffered saline (PBS, Sigma Aldrich). Cells were incubated with pre-warmed 0.05% Trypsin-EDTA (Gibco) for up to 5 mins at 37°C. Fresh medium was added to the cells to neutralise the trypsin and the cells were centrifuged at 300xg for 7 mins in a swinging bucket centrifuge. Cells were taken for counting with a haemocytometer prior to centrifugation. Cell pellets were resuspended in 10 ml complete DMEM then appropriate numbers were seeded into flasks. HEK-293T cells were maintained in cell culture treated T-175 flasks (Corning), whilst

the other cell lines were maintained in cell culture treated T-75 flasks (Corning). All cells were incubated at 37°C with 5% CO_2.

For long term storage, cells were frozen in liquid nitrogen at low passage numbers. When freezing cells for storage, a flask of cells was trypsinised and centrifuged as above and the cell pellet was resuspended in a 90% FBS, 10% DMSO freezing medium at a density of approximately 6 million cells per ml. Cells were aliquoted into cryovials and the temperature gradually reduced through placing the vials into a freezing container containing 100% isopropanol then freezing in the -80°C freezer for at least 12 hrs before transferring the cryovials into liquid nitrogen.

2.6.2. Transfection and production of pseudotyped virus particles

HEK-293T cells were seeded 24 hrs prior to transfection in a 10 cm^2 cell culture treated dish (Corning Primaria) at a density of 1.2 x 10^6. A plasmid mix was produced containing 2 µg each of the MLV-*gag/pol*, luciferase reporter, and envelope glycoprotein plasmids in a volume of 300 µl Optimem. 24 µl linear polyethylenimine (PEI) (1 mg/ml, Polysciences) transfection reagent was added to 276 µl Optimem (Gibco) and then combined with the plasmid mix and incubated at room temperature for at least 30 mins before being added to the cells. Medium on the cells was removed and replaced with Optimem immediately prior to transfection. The medium was replaced with DMEM 6 hrs post transfection. Cell supernatant was harvested and filtered through a 0.45 µm filter 72 hrs post transfection and stored at 4°C for up to 2 weeks or aliquoted and frozen at -20°C for up to one month.

2.6.3. Pseudotype optimisation

An optimisation matrix of MLV-*gag/pol* and envelope glycoprotein plasmid amounts was performed for certain lyssavirus strains. A starting amount of 8 µg was used for each plasmid and serially diluted 5-fold. 2 µg luciferase reporter was added to each dilution of MLV-*gag/pol* prior to the addition of the envelope glycoprotein plasmid dilution. PEI was added and cells incubated and harvested as per the standard transfection protocol.

2.7 Infectivity assays

BHK-21 cells were seeded at a density of 2 x 10^4 cells per well in a white opaque cell culture treated 96-well plate (Corning) 24 hrs prior to infection. Medium was aspirated from the cells and 100 µl aliquots of pseudotyped virus was added to each well in triplicate. 4 – 6 hrs later, 100 µl DMEM was added to each well and the cells incubated for a further 72 hrs. The medium was then removed, and the cells were lysed with 50 µl cell lysis solution (Promega). 50 µl luciferase substrate (Promega) was injected into each well immediately prior to reading. Luminescence was measured using a Fluostar Omega luminometer. The optical gain was set to 3,600 as standard and reduced to 2,000 where required due to high levels of luminescence outside the linear range of detection.

2.7.1. Host tropism infectivity assays

Cells were seeded into white opaque 96-well plates 24 hrs prior to infection at a density of 2 x 10^4 for BHK-21 cells and 1.5 x 10^4 for the CRFK, RoNi/7.1 and DesNi cells. Cell seeding densities for BHK-21 and CRFK cells were based on recommendations provided by the European Collection of Authenticated Cell Cultures (ECACC). The bat cell lines RoNi/7.1 and DesNi have no recommendations, so were used at the same density as the CRFK cells to prevent over-confluence and cell detachment.

Pseudotyped viruses were previously tested on BHK-21 cells with serial dilutions for highly infectious strains in order to calculate appropriate dilutions for each pseudotyped virus. Highly infectious pseudotypes were diluted with DMEM to produce approximately 150,000 RLU when infecting BHK-21 cells and read at a gain of 3,600. Low infectivity pseudotyped viruses were used neat. 100 µl of each pseudotype or pseudotype dilution was added to the cells and infectivity assays carried out as standard. All results were read at an optical gain of 3,600 with the exception of the CRFK cells which were read at 2,000, due to high levels of luminescence.

2.8 Neutralisation assays

BHK-21 cells were seeded at a density of 2 x 10^4 per well in a white, opaque 96-well plate 24 hrs prior to neutralisation assays were performed. All serum samples were heat inactivated prior to use at 56°C for 30 mins. For single-point neutralisations, pseudotype aliquots of 100 µl were treated in triplicate with dilutions of 1:50 and 1:100 human serum or 1:100 mouse serum. For neutralisation curves, serum was diluted to 1:50 (human serum) or 1:100 (non-human primate serum) final concentrations in PBS then further diluted in a 2-fold or 5-fold serial dilutions. 25 µl of each serum dilution was added to 75 µl of pseudotyped virus and incubated at room temperature for 1 hr prior to the addition to cells. 4 – 6 hrs later, 150 µl of DMEM containing 1% penicillin/streptomycin was added to the cells. The cells were incubated for 72 hrs before lysis and luminescence measured as per the infectivity assays. Pseudotype infectivity was calculated as a percentage relative to the maximum infectivity (pseudotyped virus only) and minimum infectivity (ΔEnv pseudotypes lacking a surface glycoprotein) controls. The serum dilution required to reduce infectivity to 50% was calculated using Graphpad Prism.

2.9 Antibodies for immunoblotting

Several antibodies were used for immunoblotting to test for protein expression. Two mouse monoclonal antibodies were used to target the RABV glycoprotein, 24-3F-10 (Sigma Aldrich) and Rab-50 (Abcam). A goat anti-GAPDH (Sigma Aldrich) was used to target the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Rabbit anti-mouse HRP (Sigma Aldrich) and rabbit anti-goat HRP (Sigma Aldrich) were used as secondary antibodies.

2.9.1. Cell lysis

Transfected cells used for the production of pseudotyped particles were saved after harvesting the supernatant and then lysed to extract whole-cell proteins. Cells were lysed by adding 1 ml cell lysis buffer (Appendix II) to each 10 cm^2 dish, scraping cells from the bottom of the dish with a pipette tip then placing on a rocker for 10 mins. The lysate was then collected and centrifuged at 12,000xg for 5 mins. The supernatant was then collected and stored at -20°C.

2.9.2. Dot blot

Cell lysates were prepared for blotting by adding either a reducing or non-reducing loading buffer (Appendix II). The samples in the reducing buffer were heated to 95°C for 5 mins to denature the proteins, whilst the non-reduced samples were not.

A polyvinylidene difluoride (PVDF) membrane was cut into strips and activated in 100% methanol for 5 secs before transferring the membrane to a tank of transfer buffer (Appendix II) to equilibrate for 2-3 mins. The membrane was then removed from the tank and 2 µl cell lysate were directly spotted onto the membrane, then left to dry overnight to allow the proteins to bind.

The membrane was then re-wetted in 100% methanol and blocked in a 10% milk-PBS-TWEEN solution (Appendix II) for 1 hr. Each membrane strip was then incubated with primary antibody diluted in a 5% milk-PBS-TWEEN solution overnight.

The primary antibody solution was then removed and the membrane washed three times for 5 mins with PBS-TWEEN (Appendix II) before incubating with secondary antibody diluted in a 5% milk solution for 1 hr.

The membrane was then washed another three times with PBS-TWEEN and 300 µl horseradish peroxide substrate (Radiance Plus, Azure Biosystems) added to each membrane strip immediately prior to imaging with the Genebox chemiluminescence and fluorescence imager.

2.10 RNA extraction for gene expression analysis

6 million cells were harvested and pelleted and RNA extraction was performed using the RNEasy minikit (Qiagen) on ice. Briefly, the cells were lysed then mixed with 1 volume 70% ethanol. Lysed cells were added to a column and washed with buffers RW1 and RPE. Columns were centrifuged at max speed for 1 min to dry the membrane. RNA was eluted in 50 µl RNase-free water. RNA was quantified using a Nanodrop spectrophotometer and stored at -80°C.

2.10.1. cDNA production

RNA was converted to cDNA using EcoDry random hexamers (Takara Clontech). 3 µg RNA was added to 20 µl RNase-free water, then added to the lyophilised random hexamers. The reaction mix was then incubated at 42°C for 1 hr, then terminated by heating to 70°C for 10 mins. cDNA was stored at -20°C.

2.10.2. Primer design for qPCR

qPCR primers for the housekeeping genes *GAPDH* and *HPRT1* (hypoxanthine phosphoribosyltransferase 1)were identified from the literature, with the *GAPDH* primers being taken from a study where they were used on BHK-21 cells [200] and the *HPRT1* primers from a study where they were specifically designed to be universal primers that could be used against a range of cell lines from different animal species [201].

Primers for the three receptor genes (*CHRNA1, NCAM1* and *NGFR*) were designed based on multiple sequence alignments (Fig 2.3). Gene sequences were taken from Ensembl and Genbank and aligned using MUSCLE (Table 2.1). Primers were designed against conserved regions of the genes and were double-checked against the full annotated human gene sequences from Ensembl to ensure the primers were situated on different exons. This ensured that the size of the final PCR product would indicate whether there was any genomic DNA contamination.

2.10.3. SYBR green qPCR

cDNA was used in SYBR green qPCR reactions. The reaction mix contained 10 µl 2x SYBR Green PCR Master Mix (Applied Biosystems), 0.8 µl forward and reverse primers, 1 µl cDNA and 7.4 µl nuclease-free water. The PCR cycling conditions were as follows:

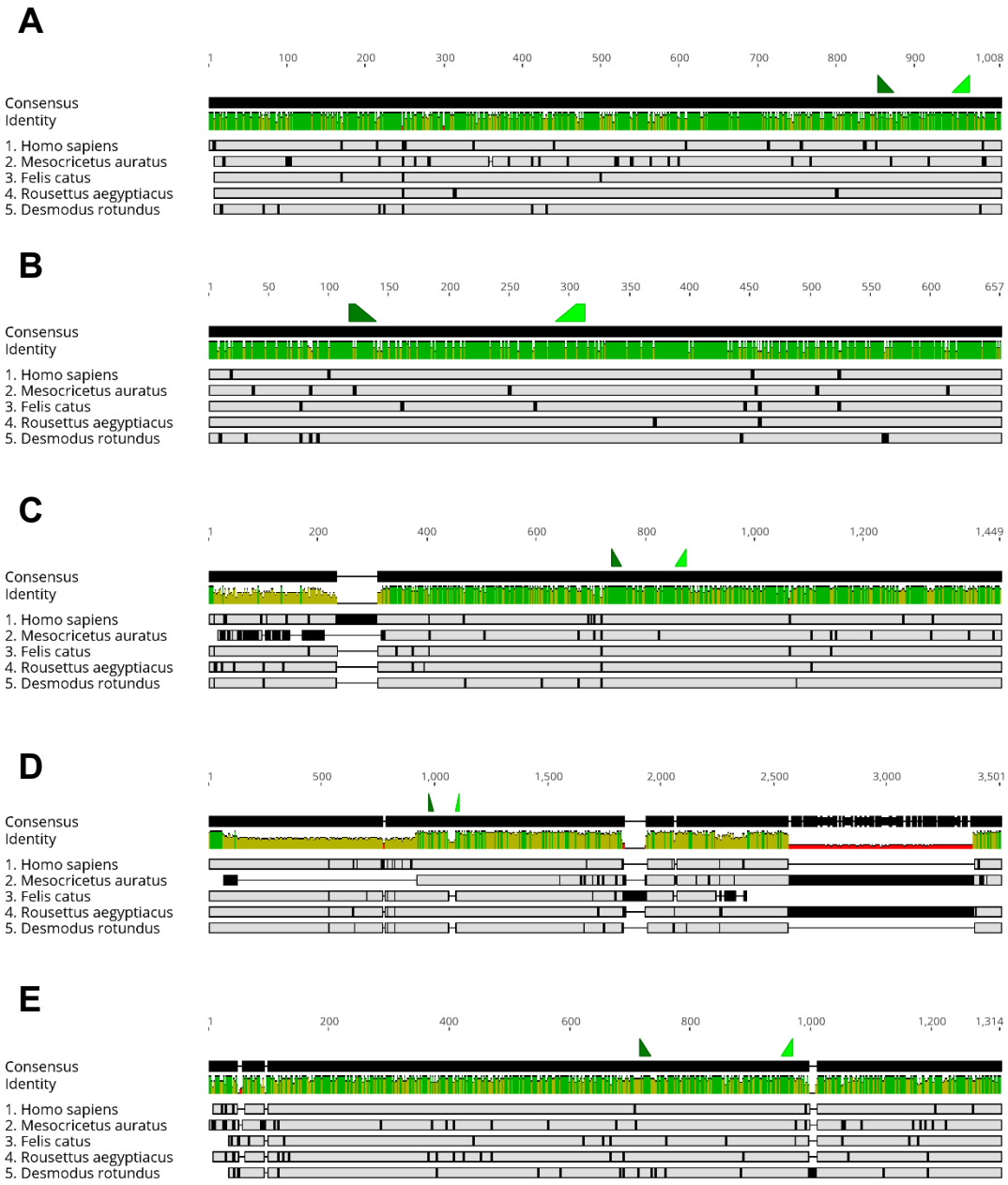


Figure 2.3 Multiple sequence alignments for qPCR genes
Multiple sequence alignments for the genes A) *GAPDH*, B) *HPRT1*, C) *CHRNA1*, D) *NCAM1* and E) *NGFR*. Primers are indicated by green triangle above the consensus sequence. Nucleotide identity is shown with colours ranging from red to green. Black highlights on individual genes show where amino acid residues differ from the consensus sequence.

Table 2.1 Gene accession numbers for genes used in multiple sequence alignments

Gene	Species	Genbank/Ensembl	Accession number
GAPDH	Homo sapiens	Ensembl	ENST00000229239.10
GAPDH	Mesocricetus auratus	Genbank	XM_021230111
GAPDH	Felis catus	Ensembl	ENSFCAT00000006876.5
GAPDH	Rousettus aegyptiacus	Genbank	XM_016162843.1
GAPDH	Desmodus rotundus	Genbank	XM_024579516.1
HPRT1	Homo sapiens	Ensembl	ENST00000298556.8
HPRT1	Mesocricetus auratus	Ensembl	ENSMAUT00000000179.1
HPRT1	Felis catus	Ensembl	ENSFCAT00000062076.2
HPRT1	Rousettus aegyptiacus	Genbank	XM_016123578.1
HPRT1	Desmodus rotundus	Genbank	XM_024566785.1
CHRNA1	Homo sapiens	Ensembl	ENST00000261007.9
CHRNA1	Mesocricetus auratus	Ensembl	ENSMAUT00000023696.1
CHRNA1	Felis catus	Ensembl	ENSFCAT00000014107.6
CHRNA1	Rousettus aegyptiacus	Genbank	XM_016134779.1
CHRNA1	Desmodus rotundus	Genbank	XM_024561551.1
NCAM1	Homo sapiens	Ensembl	ENST00000316851.11
NCAM1	Mesocricetus auratus	Ensembl	ENSMAUT00000028198.1
NCAM1	Felis catus	Ensembl	ENSFCAT00000064296.1
NCAM1	Rousettus aegyptiacus	Genbank	XM_016148997.1
NCAM1	Desmodus rotundus	Genbank	XM_024570911.1
NGFR	Homo sapiens	Ensembl	ENST00000172229.8
NGFR	Mesocricetus auratus	Ensembl	ENSMAUT00000026283.1
NGFR	Felis catus	Ensembl	ENSFCAT00000066945.2
NGFR	Rousettus aegyptiacus	Genbank	XM_016158545.1
NGFR	Desmodus rotundus	Genbank	XM_024573362.1

Initial denaturation: 95°C for 2 mins

40 cycles

95°C for 5 secs

60°C for 20 secs

Denaturation: 95°C for 10 secs

Melt curve analysis from 65°C to 95°C incrementing in 0.5°C for 5 secs

Cycle threshold (C_T) values were normalised using the $2^{-\Delta\Delta CT}$ method. In brief, this involved subtracting the average C_T value of the housekeeping gene from the average C_T of the experimental gene for both the experimental and the reference samples, which produced the ΔCT experimental and ΔCT reference figures. The ΔCT reference was then subtracted from the ΔCT experimental to produce the ΔΔCT value. Due to the amount of DNA doubling in every PCR cycle, all values are therefore in log base 2, so to calculate the fold change in gene expression, 2 is raised to the power of the negative ΔΔCT value.

2.11 Figure creation

Several software packages were used for the creation of figures in this thesis. Graphs were created and analysed using Graphpad Prism 8, sequence alignments and phylogenetic trees were created with MEGA-X and Geneious, and protein models were created using Pymol 2.3.2. Cartoons were created with Biorender.com.

Chapter 3 - Development of a lyssavirus pseudotype panel

3.1 Introduction

Rabies is an encephalitic disease caused by the classical rabies virus (RABV) that primarily infects dogs, but bats and other wildlife species such as foxes and raccoons are also important reservoirs of infection [5]. Over the years, increasing numbers of non-rabies lyssaviruses have been discovered, mainly in Europe and Africa. Whilst lyssaviruses as a group are quite closely related, they also display genetic and antigenic diversity [202]. Non-RABV lyssaviruses have been mainly found in bats though they have also been seen in a diverse selection of animals including cats, shrews, civets and various livestock species [10–12,14,15]. Due to a lack of treatment options, vaccination is the primary tool for protection against lethal rabies infection.

Rabies is predominantly caused by infection with RABV however there have been some documented cases of rabies caused by infection with non-RABV lyssaviruses [33,34,37,74,203]. The impact of non-RABV lyssaviruses on rabies burden worldwide is difficult to quantify as the majority of infections occur in developing nations where surveillance can be poor [9]. When rabies cases are recorded the specific causative agent is often still unverified [13,75]. Current tests to measure rabies virus antibody levels require the use of live RABV and must therefore be carried out in high containment facilities [3,204]. Pseudotype assays have been used in an attempt to develop methods that are safer to use due to the lack of live virus and are suitable for use in areas where high containment may not be available [180].

Pseudotypes are virus-like particles which lack the ability to replicate and are made up of a retroviral core with a viral glycoprotein of interest expressed on the outer membrane. Due to their high safety profile, pseudotypes are particularly useful for working with highly pathogenic viruses and viruses that lack treatment options. Whilst pseudotypes can't be used to investigate viral replication, they can be used to study a variety of host-viral interactions such as

virus entry into cells, antibody responses and virus neutralisation. Pseudotyping has also been used in gene therapy, as a way to target specific cells [205].

Previous attempts to generate RABV pseudotypes have met with some difficulties, resulting in low transduction efficiency in comparison to vesicular stomatitis virus (VSV) pseudotypes [143,206]. Strategies such as using chimeric glycoproteins incorporating the cytoplasmic tail from VSV have been used in an attempt to increase efficiency [207]. Despite these issues, high-titre lyssavirus pseudotype assays have been documented without the need for glycoprotein modification [180,197,208].

The majority of RABV pseudotypes generated thus far have used lab-adapted strains such as CVS-11 [180,208], CVS-N2c [206], CVS-B2c [206,207] and PV [209]; whilst other lyssavirus pseudotypes have typically used glycoprotein sequences that were obtained following virus passage in cell culture [180,197,209]. Viruses that have adapted to cell culture conditions may contain mutations that are not commonly seen in circulating wild-type virus strains which could potentially lead to differences in virus behaviour [210].

The aim of the work described in this chapter was to set up a diverse lyssavirus pseudotype panel encompassing both lab-adapted and *ex vivo* lyssavirus strains by selecting suitable virus strains from Genbank and optimising pseudotype production.

3.2 Results

3.2.1. Generation of the medoid rabies pseudotype

The RABV-medoid lyssavirus sequence was identified by collaborators in ReiThera for use as the basis of a new rabies vaccine. A medoid sequence is a sequence from a dataset that shows the least amount of divergence from the other sequences within that dataset. A medoid sequence differs from a consensus sequence in that it is not an artificial sequence, but one that is present in the analysed dataset. As the RABV-medoid sequence is less divergent

than the standard RABV vaccine strains, it may be capable of protecting against all RABV strains the current vaccines can, with potentially increased protection against more distant strains (Fig 3.1). The sequence identified as the medoid was originally isolated from the brain of a dog in Iraq, though it is unclear whether this virus was directly sequenced from original brain tissue as some of the samples from the original study were passaged once on suckling mouse brain prior to sequencing [211] . The RABV-medoid strain was used as a prototype, to establish the initial conditions for pseudotyping prior to developing the full lyssavirus panel.

The RABV-medoid sequence was cloned into pcDNA3.1, a high expression plasmid, using the Topo directional cloning kit as described in section 2.3.1.

Pseudotyped virus particles expressing the medoid rabies glycoprotein were created using a three-plasmid system, with separate plasmids expressing MLV-*gag/pol* (phCMV-5349), firefly luciferase (pTG126) and the medoid rabies glycoprotein (pcDNA3.1-RABV-medoid). 2 μg of each plasmid were transfected into HEK293T cells and the supernatant, containing virus pseudotypes, was collected 72 hrs later.

In order to confirm that transfection conditions were suitable for producing maximal yields of virus pseudotypes, an optimisation matrix was performed. This involved transfecting HEK-293T cells with serial dilutions of MLV-*gag/pol* and glycoprotein plasmids whilst maintaining a fixed quantity of luciferase plasmid.

Pseudotype infectivity was determined by adding pseudotype preparations to BHK-21 cells in triplicate wells and measuring luminescence 72 hrs later. Whilst varying the plasmid amounts led to changes in pseudotype infectivity, no increase was seen over using the standard amounts of 2 μg of each (Fig 3.2).

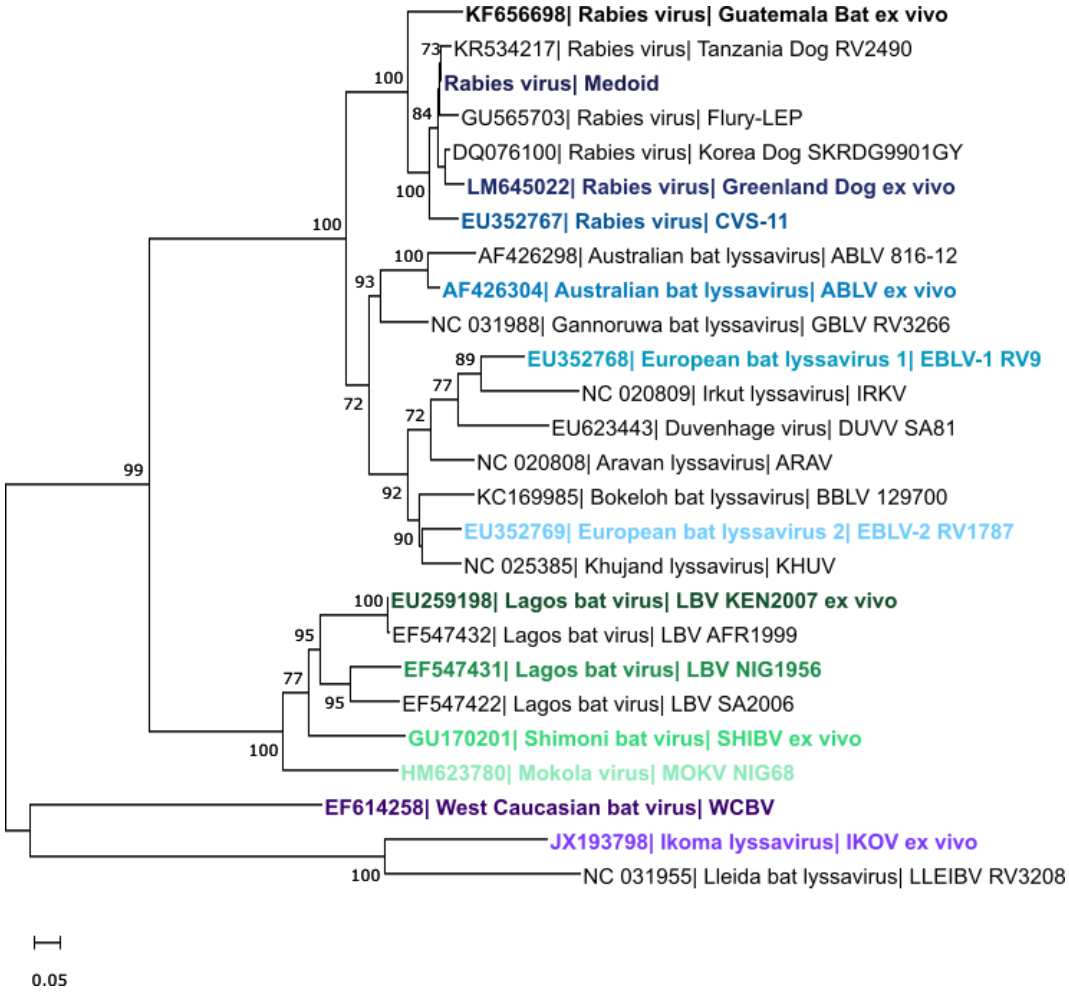


Figure 3.1 Phylogenetic tree of lyssavirus glycoprotein amino acid sequences
The maximum likelihood tree was generated using a JTT+G+I model in MEGAX. Tree topology was assessed using bootstrap analysis of 500 replicates. Bootstrap percentages are shown at relevant nodes, with values <70% not displayed. The scale bar indicates the number of amino acid substitutions per site. Coloured strains were selected for use in the lyssavirus pseudotype panel.

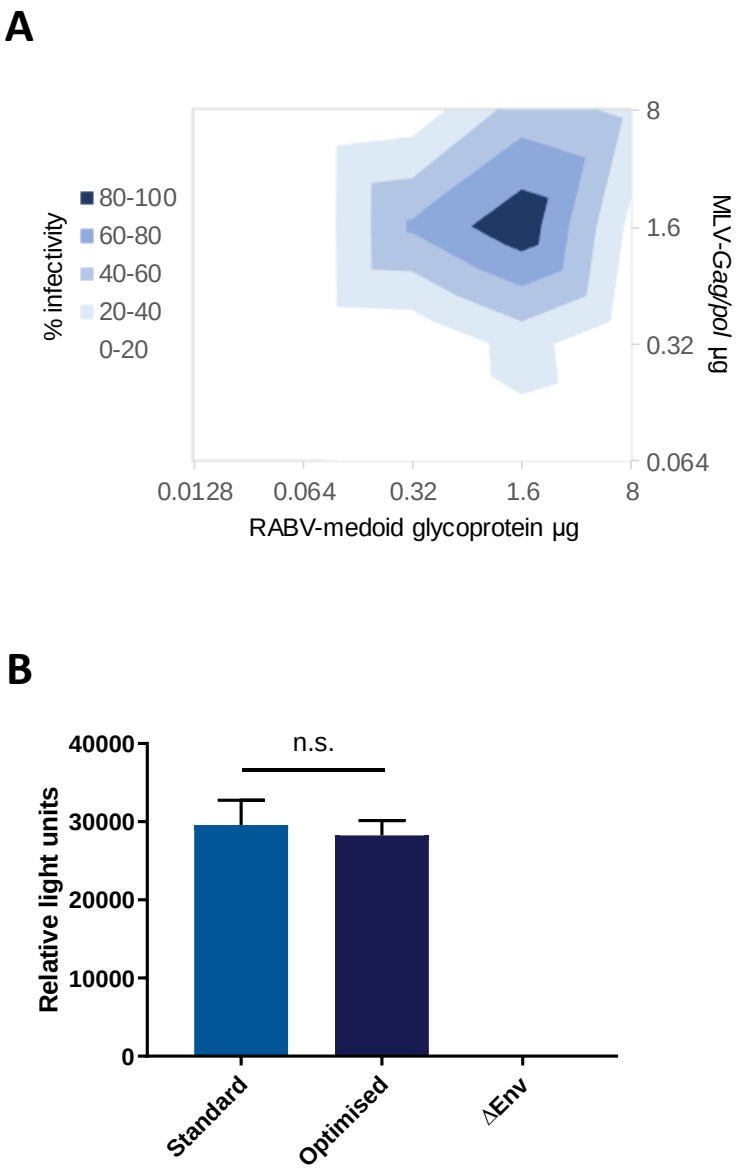


Figure 3.2 Optimisation of the production of RABV-medoid pseudotypes
A) A matrix of plasmid serial dilutions was performed to optimise the transfection and production of RABV-medoid pseudotypes. BHK-21 cells were infected with the pseudotypes in order to determine infectivity. Luminescence values were normalised between the maximal signal and the negative control, expressed as a percentage and presented as an x-y contour plot. B) Comparison between pseudotype infectivity for particles produced using 2 µg of MLV-*gag/pol* and glycoprotein plasmids (standard) and particles produced using the optimised amounts (1.6 µg of MLV-*gag/pol* and glycoprotein plasmids) as determined by the optimisation matrix (optimised). Statistical significance was assessed by one-way ANOVA. Wells were infected in triplicate and the assay was repeated 3 times, with the results presented being from 1 representative assay. Error bars indicate standard deviation.

3.2.2. MLV and HIV-1 pseudotype backbone comparison

Pseudotypes are commonly produced using both MLV and HIV-1 backbones, so to determine which system would be the most appropriate for future experiments pseudotypes were produced with both. Two HIV-1 lentiviral systems were available, pNL4-3 (Fig 3.3) and psPAX2 (Fig 3.4), and compared with the MLV system. Comparisons were performed on different days, hence the variation in RLU values shown by the MLV-based pseudotypes. The pNL4-3 HIV backbone was a two-plasmid system, with the luciferase encoded on the *gag/pol* plasmid, whilst the psPAX2 and MLV systems were both three-plasmid systems with separate reporter plasmids.

As seen in figures 3.3 and 3.4, whilst the VSV control led to lower RLU values than the RABV-medoid in the MLV system, this was reversed in both the HIV-1 systems, with VSV leading to much higher RLU values. Whilst VSV infectivity was much improved in the pNL4-3 system, the RABV-medoid was reduced, with less than 1,000 RLU. For psPAX2, on the other hand, both RABV-medoid and VSV were greatly increased.

Whilst psPAX2 led to high RLU values for both RABV-medoid and the VSV control, the presence of an innate restriction factor against HIV-1 present in bat cells precludes the use of HIV-1-based pseudotypes when testing pseudotype infectivity in bat cells [184,212]. As future experimental plans involved comparing infectivity across different cell lines including bat cells, it was decided to proceed using the MLV pseudotype system despite the lower RLU values obtained. ΔEnv values were also notably higher with the psPAX2 (~7,000 RLU) system than with MLV (~900 RLU), though still significantly lower than glycoprotein-containing pseudotypes. Whilst VSV in particular seemed low with the MLV backbone, the RLU values were on par with those seen previously with this system [182].

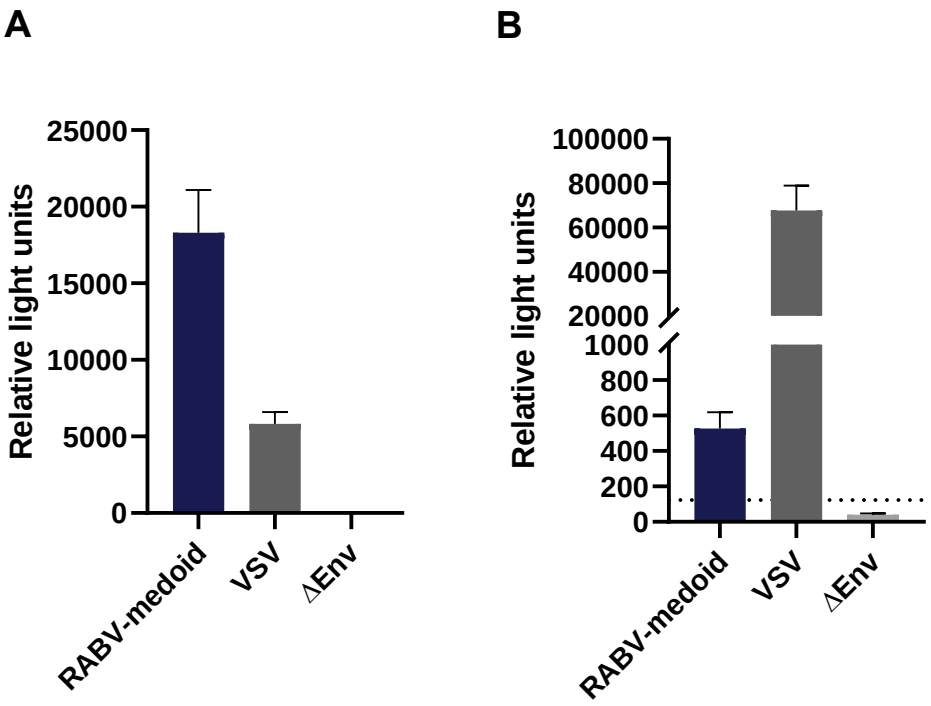


Figure 3.3 Comparison between MLV and pNL4-3 pseudotype systems
RABV-medoid and VSV pseudotypes were produced with both A) MLV and B)HIV-1 (pNL4-3) backbones and used to infect BHK-21 cells. Luminescence was measured at 3,600 optical gain for all MLV-based pseudotypes and for the RABV-medoid and ΔEnv HIV-1-based pseudotypes. The HIV-1 VSV pseudotype was measured at a reduced optical gain of 1,800. The positive threshold was defined as 3x the signal of the ΔEnv negative control and is shown with a dotted line. Wells were infected in triplicate the assay was repeated 3 times, with the results presented being from 1 representative experiment. Error bars indicate standard deviation.

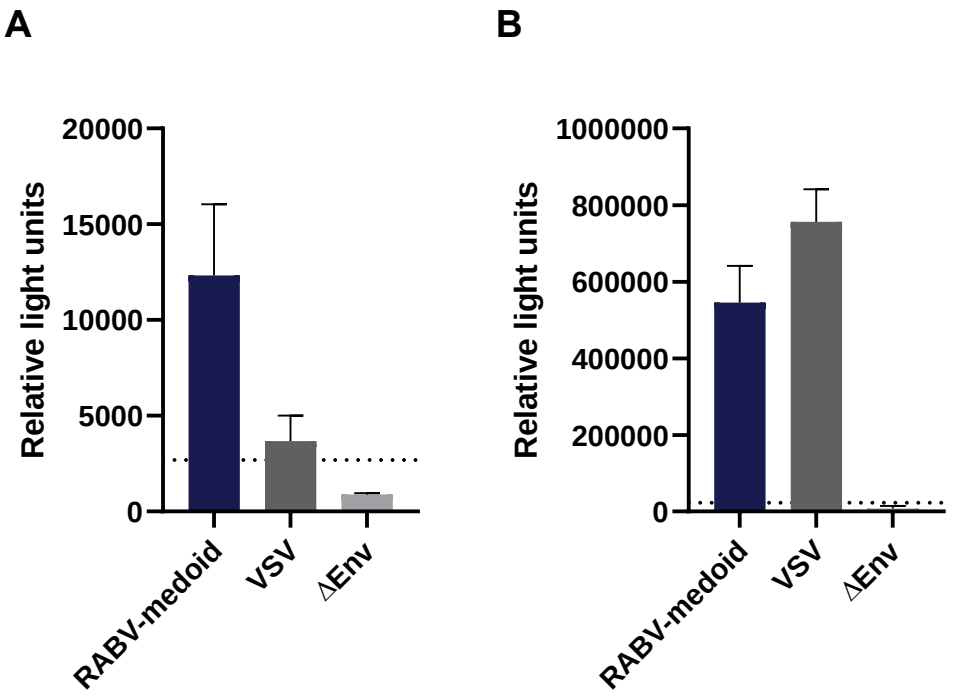


Figure 3.4 Comparison between MLV and psPAX2 pseudotype systems
RABV-medoid and VSV pseudotypes were produced with both A) MLV and B)HIV-1 (psPAX2) backbones and used to infect BHK-21 cells. Luminescence was measured at 3,600 optical gain for all MLV-based pseudotypes and for the RABV-medoid and ΔEnv HIV-1-based pseudotypes. The HIV-1 VSV pseudotype was measured at a reduced optical gain of 1,000. The positive threshold was defined as 3x the signal of the ΔEnv negative control and is shown with a dotted line. Wells were infected in triplicate the assay was repeated 3 times, with the results presented being from 1 representative experiment. Error bars indicate standard deviation.

3.2.3. Development of the full lyssavirus pseudotype panel

In generating the lyssavirus pseudotype panel, strains were selected to provide broad coverage of the lyssavirus family and included strains from each of the three lyssavirus phylogroups (Fig 3.1).

Strains that had been sequenced directly *ex vivo* from infected animal tissue were preferentially selected, however *ex vivo* sequences were not available for many lyssavirus species as the viruses were passaged in cell culture or mouse brains for 1-2 passages prior to sequencing.

Several lyssavirus strains that had been previously pseudotyped [180,197] were kindly provided by Dr Edward Wright (University of Sussex), with 2/8 of these being *ex vivo* strains [194,196]. An additional 4 *ex vivo* lyssavirus strains were identified on Genbank [98,198,199,213], and these were synthesised by Geneart Gene Synthesis (Thermo Fisher). All *ex vivo* lyssavirus strains have been labelled with the "-ev" suffix for ease of identification (Table 3.1).

To ensure that any differences in pseudotype infectivity were due to differences in the glycoprotein sequence, all lyssavirus glycoproteins were cloned into the pcDNA3.1 plasmid vector for consistency.

Initially, cloning was started using the Topo directional cloning kit, as for the RABV-medoid strain, however cloning efficiency was low with many colonies lacking glycoprotein inserts. Only 5 strains (RABV-medoid, RABV-GrD-ev, RABV-GtB-ev, RABV-CVS and LBV-ev) were successfully cloned using the Topo cloning kit, with the remainder being cloned by the InFusion cloning method as described in section 2.3.3.

Once all lyssavirus glycoproteins were cloned into the pcDNA3.1 vector, pseudotyped virus particles were created and test infections carried out to assess infectivity. Pseudotyped virus infectivity varied from 500 RLU to >2 million RLU for the SHIBV-ev lyssavirus strain. As this was greater than the limit

Table 3.1 List of lyssaviruses selected for the pseudotype panel

Virus	Strain	Alias	*Ex vivo?*	Accession
RABV	Medoid	RABV-medoid	NA	AGN94271
RABV	CVS-11	RABV-CVS	N	EU352767
RABV	Greenland dog isolate Gra 24.06-3-D	RABV-GrD-ev	Y	LM645022
RABV	Guatemala bat isolate A13-0465	RABV-GtB-ev	Y	KF656698
ABLV	661-22	ABLV-ev	Y	AF426304
EBLV-1	RV9	EBLV-1	N	EU352768
EBLV-2	RV1787	EBLV-2	N	EU352769
LBV	KE131	LBV-ev	Y	EU259198
LBV	LBV.NIG56-RV1	LBV	N	EF547431
SHIBV	SHIBV	SHIBV-ev	Y	GU170201
MOKV	MOKV.NIG68-RV4	MOKV	N	HM623780
WCBV	WCBV	WCBV	N	EF614258
IKOV	RV2508	IKOV-ev	Y	JX193798

of detection of the plate reader, this sample was therefore re-measured using a lower optical gain of 2,000 rather than the standard optical gain of 3,600 (Fig 3.5). EBLV-2 did not cross the positive threshold of three-times the signal of the ΔEnv negative control (300 RLU), whilst RABV-GrD-ev was slightly higher than the positive threshold at 500 RLU.

As the majority of the lyssavirus pseudotypes led to low luminescence output, with a limited dynamic range between the pseudotype and the ΔEnv negative control, optimisation matrices were performed on three *ex vivo* virus strains, RABV-GrD-ev, ABLV-ev and LBV-ev, in an attempt to increase pseudotype infectivity (Fig 3.6). Adjusting the plasmid amounts transfected into cells did not increase infectivity for the RABV-GrD-ev pseudotype, which was the least infectious of the three pseudotypes to begin with. There was a notable, but non-significant, increase in infectivity for the ABLV-ev pseudotype from 2,000 RLU to 6,000 RLU and a statistically significant increase in infectivity for the LBV-ev pseudotype, from 2,000 RLU to 15,000 RLU.

3.2.4. Site-directed mutagenesis and cloning into plasmid vector pI.18

Whilst optimisation of plasmid transfection amounts could lead to improvements in pseudotype infectivity, the impact varied between virus strains, as shown in figure 3.6. The lyssavirus strains provided by Dr Edward Wright had all been successfully pseudotyped previously with high titres [180,197], however these pseudotypes used glycoproteins that were cloned into the pI.18 pUC-based plasmid vector, rather than pcDNA3.1. Therefore, in order to clone the RABV-medoid and commercially synthesised *ex vivo* lyssavirus strains into the pI.18 vector, site-directed mutagenesis (SDM) was performed on the pI.18 plasmid.

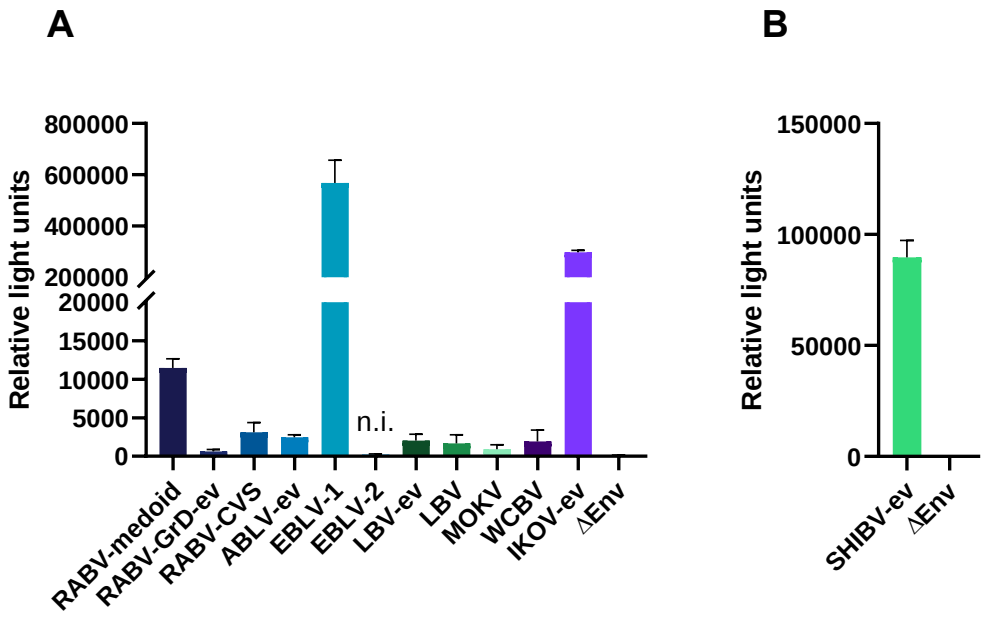


Figure 3.5 Infectivity of the lyssavirus pseudotypes

Lyssavirus pseudotypes were used to infect BHK-21 cells and luminescence measured in RLU at A) 3,600 optical gain and B) 2,000 optical gain, with the sensitivity of the sensor being reduced where light output fell outside of the linear range of detection for particularly infectious pseudotypes. The positive threshold was set at 3x the signal from the ΔEnv negative control. Wells were infected in triplicate and the assay was repeated 2 times, with the results presented being from 1 representative experiment. Error bars indicate standard deviation.

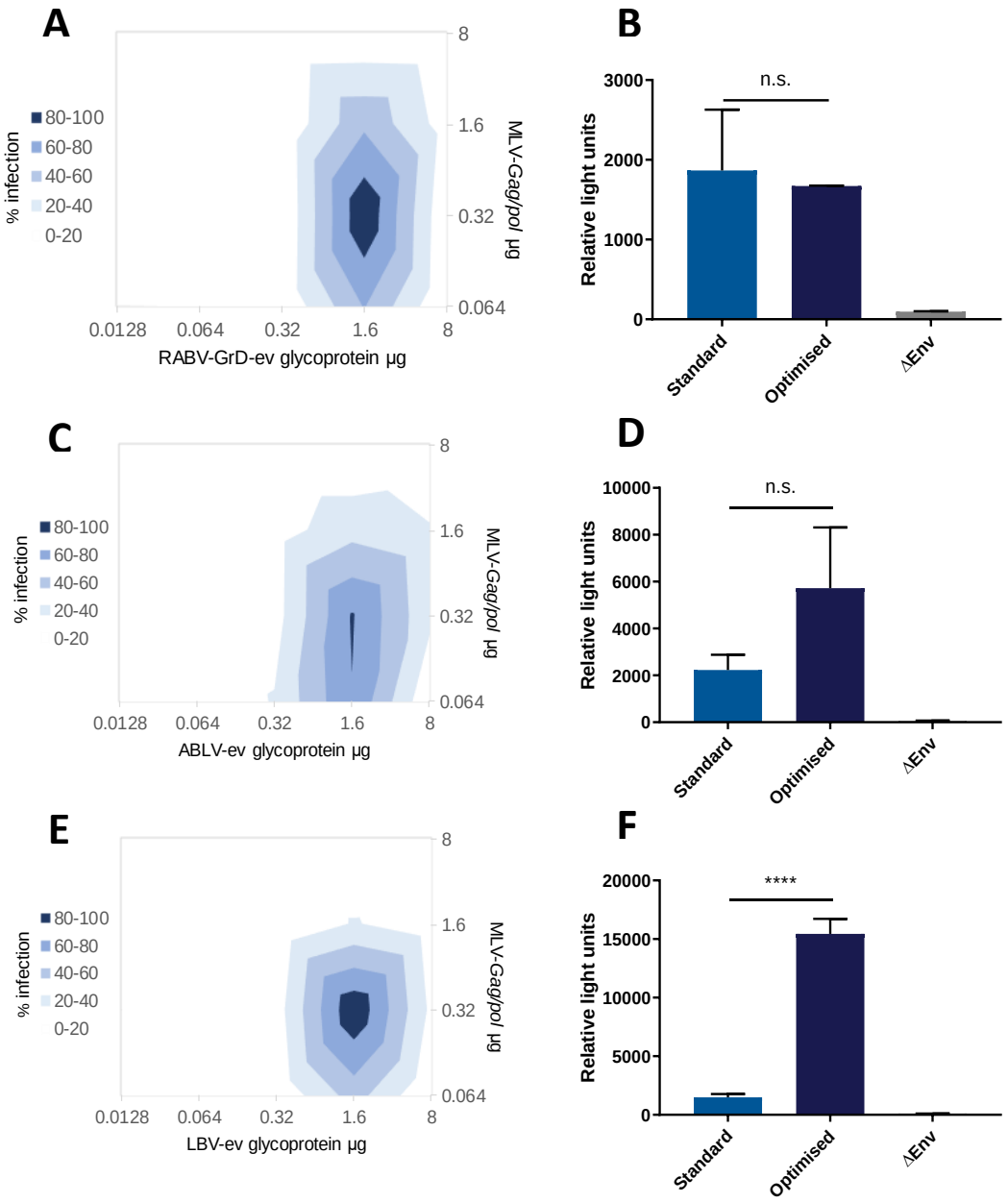


Figure 3.6 Optimisation of three *ex vivo* lyssavirus pseudotypes
Optimisation matrices were created for the lyssaviruses A) RABV-GrD-ev, C) ABLV-ev and E) LBV-ev. Luminescence values were normalised between the maximal signal and the negative control, expressed as a percentage and presented as x-y contour plots. Infectivity data for particles produced using the standard 2 µg plasmid amounts (standard) and the optimised plasmid amounts (optimised) is shown for the lyssaviruses B) RABV-GrD-ev, D) ABLV-ev and F) LBV-ev. Wells were infected in triplicate and each optimisation matrix was produced twice, with the exception of LBV-ev where only a single optimisation matrix was produced. Statistical significance was assessed by one-way ANOVA. Error bars indicate standard deviation.

The SDM of pI.18 was performed in order to fulfil three aims: to remove the lyssavirus glycoprotein present in the vector in order to produce an empty plasmid; to remove the EcoRI restriction enzyme site that was in the multiple-cloning site; and to insert a new EcoRI restriction enzyme site flanked by a 15 bp region correlating with the pcDNA3.1 multiple-cloning site. This was in order to simplify the InFusion cloning method by allowing the same cloning primers to be used for cloning into both pcDNA3.1 and pI.18 for any individual glycoprotein. SDM was carried out as described in section 2.5.

Once the pI.18 plasmid was mutated, the RABV-medoid and commercially synthesised *ex vivo* lyssavirus strains were cloned into it by InFusion cloning. The resulting plasmids were then used to produce pseudotyped virus particles and test infections performed on BHK-21 cells (Fig 3.7).

Cloning the lyssavirus glycoproteins into pI.18 led to an increase in infectivity to such an extent that RLU values fell outside the limit of detection when measured at a standard optical gain for many lyssavirus strains. There were only three lyssavirus strains that showed reduced infectivity in the pI.18 plasmid in comparison to the pcDNA3.1: RABV-medoid, SHIBV-ev and IKOV-ev (Fig 3.7), though all were above the positive threshold. These three pseudotypes still had good luminescence output with pI.18, whilst the other lyssaviruses were mostly poor in pcDNA3.1, so the pI.18 plasmid was used as the glycoprotein vector for all lyssavirus pseudotyping from this point on.

3.2.5. Site-directed mutagenesis of the RABV-GtB-ev lyssavirus strain

When selecting *ex vivo* lyssavirus strains for inclusion in the pseudotype panel, two RABV strains were chosen, one isolated from a dog (RABV-GrD-ev) and one from a bat (RABV-GtB-ev), in order to represent two major RABV reservoirs.

The bat RABV strain was not infectious in pcDNA3.1 therefore an optimisation matrix was carried out to see if this would increase infectivity. Whilst RLU values were slightly increased when the plasmid amounts were varied, they did

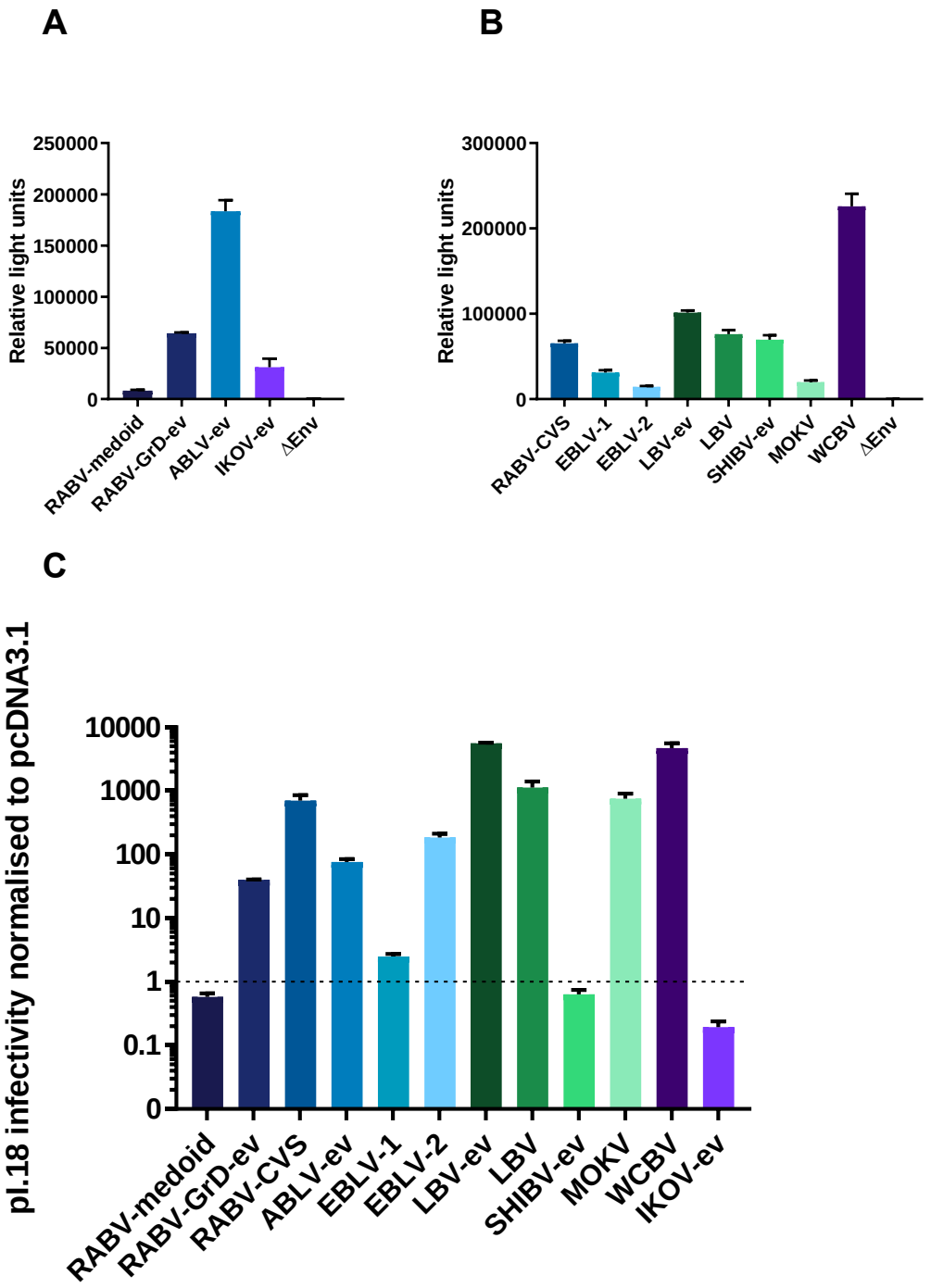


Figure 3.7 Lyssavirus infectivity is increased with the pI.18 plasmid in comparison to the pcDNA3.1 plasmid

Lyssavirus pseudotypes were produced with pI.18 plasmids containing the lyssavirus glycoprotein sequences. BHK-21 cells were infected with the resulting pseudotypes and luminescence read at A) 3,600 optical gain and B) 2,000 optical gain. The positive threshold was set to 987 RLU, 3x the ΔEnv negative control. C) Pseudotype infectivity was compared between pI.18 and pcDNA3.1. pI.18 pseudotype luminescence values were normalised relative to the corresponding pcDNA3.1 luminescence values measured at the same optical gain. Wells were infected in triplicate and the assay was repeated twice, with the results presented being from 1 representative experiment. Error bars indicate standard deviation.

not cross the positive threshold of three times higher than the negative control (Fig 3.8).

Whilst the dog RABV strain also had very low infectivity in pcDNA3.1, just above the positive threshold, once it was cloned into pI.18 infectivity was greatly increased. The bat RABV strain however, still showed low levels of infectivity in pI.18 where it was able to reach the positive threshold but not surpass it, particularly taking into account the standard deviation (Fig 3.9). There was some inter-assay variability, with RLU values ranging from <100 RLU to approximately 500 RLU, however values never increased over the ΔEnv significantly.

The amino acid sequence for RABV-GtB-ev was inspected and compared to the sequences for the other lyssavirus glycoproteins. All of the lyssaviruses had 14 cysteine residues in their ectodomains except for EBLV-1, which had 15 cysteine residues, and RABV-GtB-ev, which had 13 residues (Fig 3.10).

SDM was carried out on the RABV-GtB-ev lyssavirus glycoprotein in order to introduce an extra cysteine residue at position 159 (A159C), in line with the other lyssavirus strains. The mutated RABV-GtB-ev strain was only cloned into the pI.18 plasmid vector as this vector had already proven to be the most suitable for the majority of other lyssavirus strains.

Pseudotypes of the mutated RABV-GtB-ev strain were produced and test infections carried out as previously, however the addition of an extra cysteine residue did not lead to an increase in pseudotype infectivity (Fig 3.10). As previous attempts at optimisation using a matrix of plasmid concentrations had a limited impact on pseudotype infectivity, optimisation was not repeated for the RABV-GtB-ev strain cloned into pI.18 or its mutated form.

3.2.6. Protein blotting

To further investigate whether the improvement in pseudotype production when using the pI.18 plasmid instead of the pcDNA3.1 plasmid was due to a

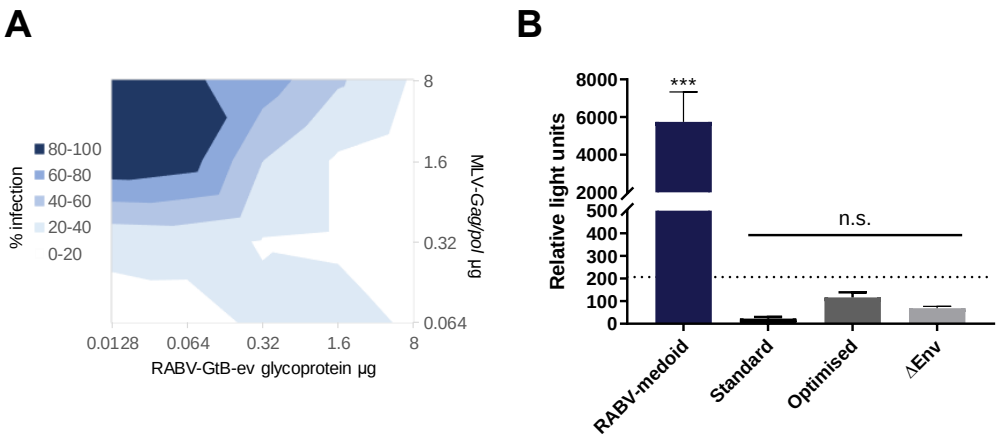


Figure 3.8 Optimisation of the production of RABV-GtB-ev pseudotypes
A) An optimisation matrix was created for the RABV-GtB-ev lyssavirus using the pcDNA3.1-GtB-ev plasmid. Luminescence values were normalised between the maximal signal and the negative control, expressed as a percentage and presented as an x-y contour plot. B) Comparison between pseudotype infectivity for particles produced using 2 µg of MLV-gag/pol and RABV-GtB-ev pcDNA3.1 plasmids (standard) and particles produced using the optimised amounts (1.6 µg MLV-*gag/pol*, 0.0128 µg RABV-GtB-ev) as determined by the optimisation matrix (optimised). RABV-medoid in pcDNA3.1 was included as a positive control. The positive threshold was set to 3x the ΔEnv signal and displayed as a dotted line. Wells were infected in triplicate and the optimisation matrix was produced twice, with the results presented being from a single representative experiment. Statistical significance was assessed by one-way ANOVA. Error bars indicate standard deviation.

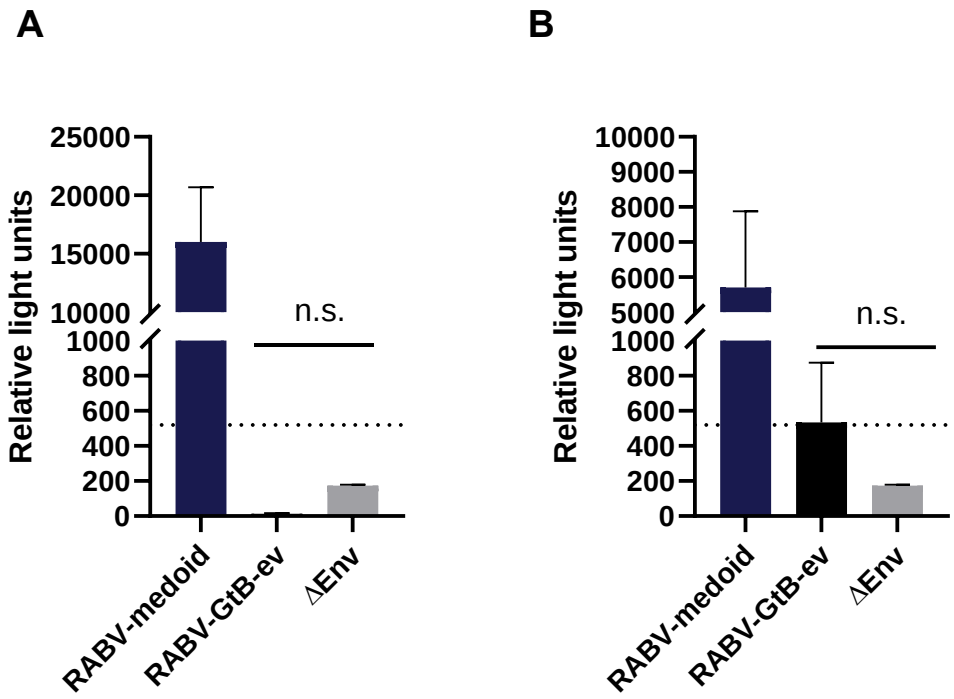


Figure 3.9 Infectivity of RABV-GtB-ev in pcDNA and pI.18
RABV-GtB-ev pseudotypes were produced using the expression plasmids A) pcDNA3.1 and B) pI.18. RABV-medoid was included as a positive control using the same expression vectors. The positive threshold was set to 3x the signal of the negative control ΔEnv and displayed as a dotted line. Wells were infected in triplicate and the assay was repeated twice, with the results presented being from a single representative experiment. Statistical significance was assessed by one-way ANOVA. Error bars indicate standard deviation.

A

	SP				Ectodomain														TM					CD	
	-15	-9	-6	-3	24	35	61	94	159	168	169	189	207	223	228	252	283	344	351	455	456	458	460	461	464	465
RABV-medoid	A	L	F	**C**	**C**	**C**	**C**	**C**	**C**	Y	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	I	F	M	**C**	**C**	V	N
RABV-GrD-ev	A	L	F	**C**	**C**	**C**	**C**	**C**	**C**	Y	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	I	F	M	**C**	**C**	G	N
RABV-CVS	V	L	F	**C**	**C**	**C**	**C**	**C**	**C**	Y	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	F	S	M	W	**C**	A	N
RABV-GtB-ev	V	L	S	**C**	**C**	**C**	**C**	**C**	A	Y	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	I	**C**	I	**C**	**C**	A	K
ABLV-ev	I	L	F	**C**	**C**	**C**	**C**	**C**	**C**	F	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	I	I	A	**C**	**C**	**C**	K
EBLV-1	T	F	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	I	I	A	**C**	**C**	F	K
EBLV-2	A	L	L	**C**	**C**	**C**	**C**	**C**	**C**	F	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	I	F	A	**C**	**C**	V	K
LBV-ev	F	**C**	I	S	**C**	**C**	**C**	**C**	**C**	S	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	L	R	**C**	**C**	M	R
LBV	N	**C**	I	S	**C**	**C**	**C**	**C**	**C**	S	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	L	R	**C**	**C**	R	G
SHIBV-ev	**C**	**C**	I	S	**C**	**C**	**C**	**C**	**C**	S	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	L	**C**	**C**	**C**	R	R
MOKV	**C**	I	T	S	**C**	**C**	**C**	**C**	**C**	S	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	L	R	**C**	**C**	V	G
WCBV	F	G	M	S	**C**	**C**	**C**	**C**	**C**	Y	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	L	F	G	**C**	**C**	**C**	R
IKOV-ev	V	G	L	G	**C**	**C**	**C**	**C**	**C**	F	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	**C**	W	F	V	L	L	V	**C**

B

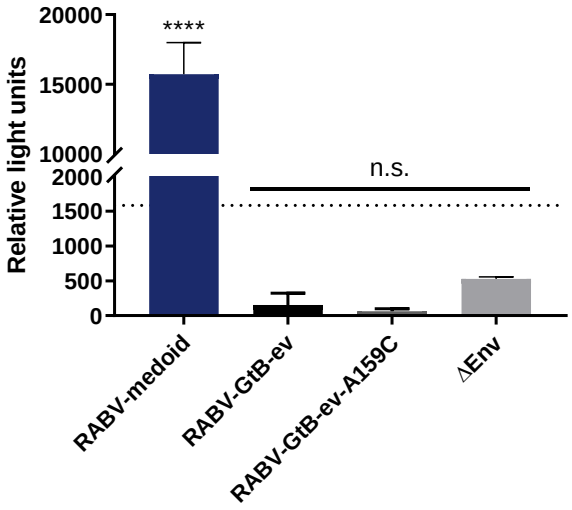


Figure 3.10 Site-directed mutagenesis of RABV-GtB-ev was unable to increase pseudotype infectivity

A) Schematic showing the locations of all cysteine residues within the lyssavirus glycoprotein genes. Amino acid numbering starts from the first amino acid following the signalling peptide. Amino acid numbering within the signal peptide ranges from -19 to -1. (SP: signal peptide, TM: transmembrane domain, CD: cytoplasmic domain) B) Pseudotypes were produced of the RABV-GtB-ev and mutated A159C strains using the pI.18 plasmid. BHK-21 cells were infected and luminescence read at 3,600 optical gain. RABV-medoid in pI.18 was included as a positive control. The positive threshold was set at 3x the negative control and displayed as a dotted line. Wells were infected in triplicate and the assay was repeated twice, with the results presented being from a single Statistical significance was determined by one-way ANOVA. Error bars indicate standard deviation.

difference in protein expression levels, protein blotting experiments were carried out.

Two antibodies targeted against the RABV glycoprotein were sourced (24-3F-10 and Rab50) along with a GAPDH antibody as a positive control. Cell lysates were taken from cells transfected with pI.18-RABV-CVS or ΔEnv as a negative control. Dot blots were then performed on the cell lysates in order to test different antibody concentrations and conditions.

Whilst the GAPDH antibody was able to detect protein in both reducing and non-reducing conditions, neither of the two anti-RABV glycoprotein antibodies gave a signal at all (Fig 3.11 and 3.12).

3.3 Discussion

Pseudotyping is a flexible tool that can be used for a wide range of applications whilst avoiding the safety concerns associated with working with live, replicating viruses. A panel of lyssavirus pseudotypes encompassing both *ex vivo* and lab adapted strains has been developed through the use of site-directed mutagenesis and InFusion cloning.

Infectivity of the lyssavirus pseudotypes varied considerably between strains, with the RABV-medoid, RABV-GrD-ev, ABLV-ev and IKOV-ev strains proving to be less infectious than the other lyssaviruses. Luminescence for these pseudotypes therefore needed to be measured at a higher optical gain, increasing the sensitivity of the plate reader's sensor to light output. It is interesting to note that all of the lyssaviruses showing reduced infectivity are *ex vivo* strains, with the exception of RABV-medoid, which has an unknown passage history. There were two *ex vivo* lyssaviruses in the highly infectious category (LBV-ev and SHIBV-ev) but the majority were lab-adapted or previously passaged strains. This could indicate that growing these viruses in cell culture has led to subtle changes in their glycoprotein sequences to adapt to growth in cell culture conditions, and thus increased the infectivity of their corresponding pseudotypes.

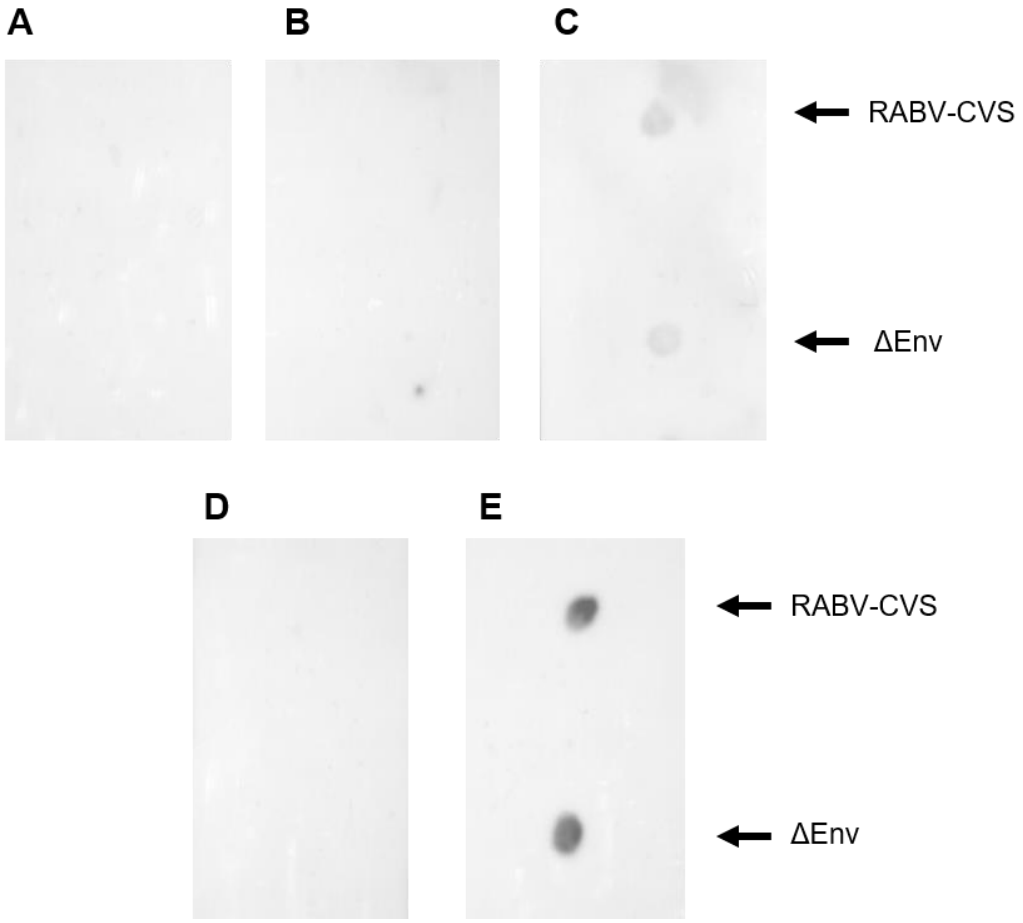


Figure 3.11 Dot blots using the anti-RABV glycoprotein antibody 24-3F-10
Cell lysates from cells used to produce RABV-CVS and ΔEnv pseudotypes were examined for protein expression using the anti-RABV glycoprotein antibody 24-3F-10 at A) 1/500 dilution and B) 1/250 dilution, with C) anti-GAPDH at 1/6000 dilution as a positive control. Cell lysates were also probed under non-reducing conditions at D) 1/500 dilution and with E) GAPDH at 1/6000 as a positive control. The dot blots were only performed a single time.

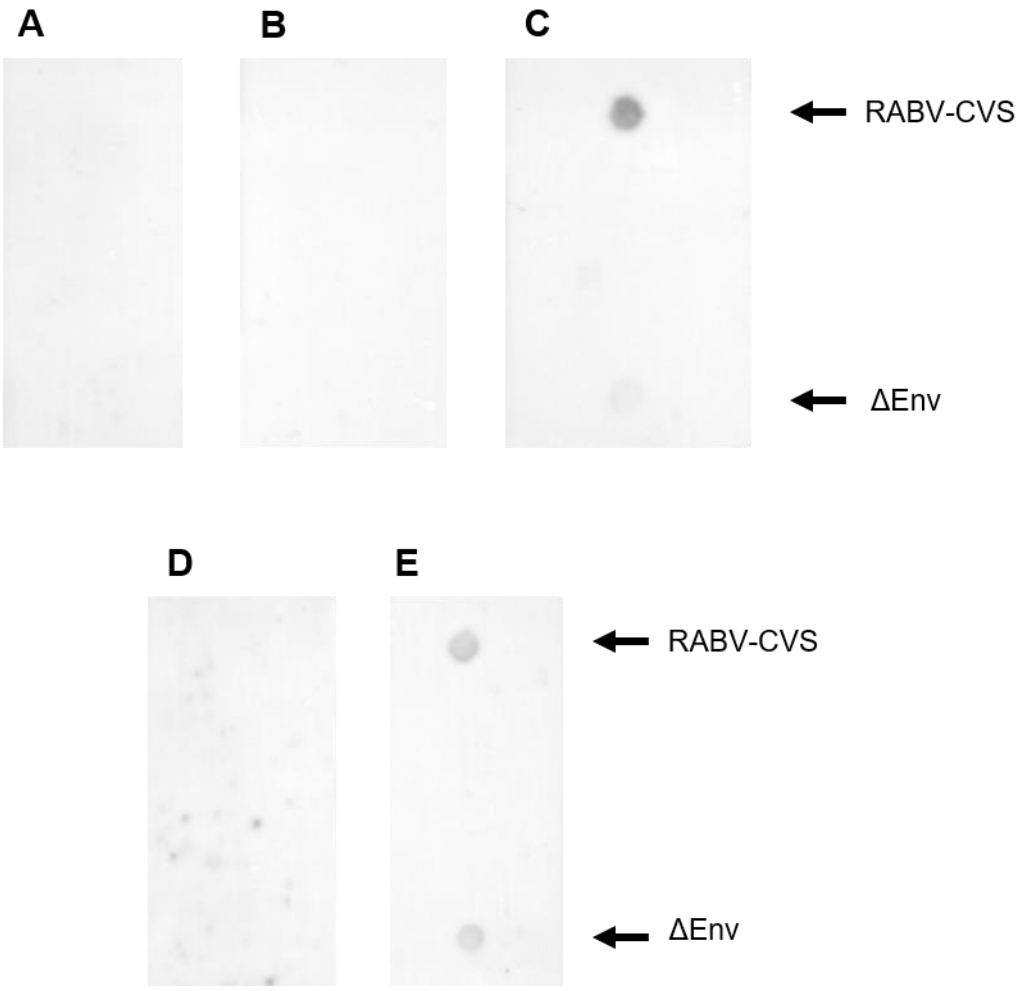


Figure 3.12 Dot blots using the anti-RABV glycoprotein antibody Rab50
Cell lysates from cells used to produce RABV-CVS and ΔEnv pseudotypes were examined for protein expression using the anti-RABV glycoprotein antibody Rab50 at A) 1/500 dilution and B) 1/250 dilution, with C) anti-GAPDH at 1/6000 dilution as a positive control. Cell lysates were also probed under non-reducing conditions at D) 1/500 dilution and with E) GAPDH at 1/6000 as a positive control. The dot blots were only performed a single time.

Both positive and negative controls were used to ensure that transfection was working as expected. VSV was used as a positive control as it has been used extensively in multiple pseudotype systems and is known to be easily pseudotyped with broad host tropism, whilst ΔEnv was used as a negative control to determine the lower level of infectivity caused by non-specific particle uptake into cells.

It is widely known that some viruses are difficult to grow in cell culture, and this can correlate with difficulty in producing virus pseudotypes. Hepatitis C in particular is known to yield very low titres of pseudotypes when using virus strains isolated from patients, leading studies to focus on strains that are easy to manipulate in the lab [182]. This evidence suggests that adaptation to cell culture conditions can affect a virus' phenotypic properties. Studies of rabies virus evolution however have consistently predicted that RABV undergoes strong purifying selection, which may lead to limited mutation rates [97,214–216]. Previous research has shown that after a limited number of passages in cell culture, RABV shows only a few mutations at the quasispecies level, and none in the consensus sequence [217]. This may indicate that lyssavirus adaptation progresses at a slower rate than that seen by other RNA viruses.

The lower levels of infectivity demonstrated by the *ex vivo* lyssavirus strains in this study may be caused by a lack of adaptation to cell culture conditions or may be mere coincidence. Ideally, paired sequences from closely related lyssavirus strains taken *ex vivo* and from lab-adapted viruses would be used to further investigate this. At the start of this investigation there were very few *ex vivo* sequences available for any lyssavirus, with the exception of RABV. As high-throughput sequencing methods become more common-place, it is likely that the majority of virus sequences will eventually be taken directly from an infected host, rather than through traditional virus isolation and expansion techniques.

One way to increase pseudotype infectivity is through optimisation of plasmid transfection into HEK-293T producer cells. Some viral glycoproteins such as

Ebola virus glycoprotein are toxic to cells [218], so high expression of the protein may lead to a reduction in pseudotype yield. The RABV glycoprotein has been previously reported to cause apoptosis in cells exposed to high concentrations of the protein [100], therefore adjusting the amounts of plasmids transfected into cells may increase cell survivability and lead to a higher concentration of pseudotyped virus particles.

Optimisation matrices were performed on several lyssavirus strains in this study (RABV-medoid, RABV-GrD-ev, RABV-GtB-ev, ABLV-ev and LBV-ev), to varying results. Pseudotype infectivity was increased substantially for the LBV-ev strain, but the impact on other strains was negligible, which may indicate that glycoprotein toxicity is not a dominating factor in the production of lyssavirus pseudotypes.

The pseudotype core used in the creation of pseudotyped viruses is also known to have an impact on pseudotype infectivity. Both Hepatitis C and Influenza H5N1 have been shown to be more efficiently pseudotyped with MLV backbones than with HIV-1 [182,219]. RABV and lyssavirus pseudotype studies have commonly used HIV-1 as the pseudotype backbone and these pseudotypes have proven useful for looking at neutralisation, antigenic sites and neuronal tropism [140,187,209].

Two HIV-1 pseudotype systems were tested in comparison to MLV for pseudotyping the RABV-medoid strain in comparison to VSV. Both HIV-1 systems led to substantially higher VSV infectivity than RABV-medoid, with reduced optical gains being required to measure luminescence. The MLV-based pseudotypes on the other hand had VSV RLU values slightly lower than those from RABV-medoid, though they were on par with those seen previously using this system [182].

The pNL4-3 HIV pseudotypes had very low RLU values for the RABV-medoid strain in comparison to the MLV pseudotypes despite the increase in VSV infectivity, though they still surpassed the positive threshold of three times

greater than the negative control. pNL4-3 has been previously noted to have low pseudotype yield which may account for the limited infectivity seen in this test [208].

The psPAX2 HIV-1 pseudotypes however showed an increase in RLU values for both RABV-medoid and VSV. These results are similar to those previously seen in another study which compared MLV and HIV-1 pseudotype backbones for the production of lyssavirus pseudotypes [180]. The previous study showed that HIV-1-based RABV pseudotypes had higher viral titres than the MLV-based RABV pseudotypes with a luciferase reporter, whilst MLV-based RABV pseudotypes were more infectious than HIV-1-based RABV pseudotypes with a GFP reporter. The cell line tested also had an impact on pseudotype titre, with BHK-21 cells showing an increased permissiveness for HIV-1-based pseudotypes regardless of reporter gene used, whilst N2A cells could only be infected by MLV-based pseudotypes [180].

Whilst the planned neutralisation experiments would be performed on BHK-21 cells, as these are the standard cell line used for RABV neutralisation assays, host species tropism experiments would require infectivity assays on a variety of different cell lines. As HIV-1-based pseudotypes are not infectious in bat cells [184], MLV-based pseudotypes would be required for these experiments. Therefore, whilst the psPAX2 lentiviral system led to more infectious pseudotypes overall, the MLV system was the one chosen to take forward with the production of the lyssavirus pseudotype panel.

Selection of the plasmid vector that the glycoproteins were cloned into also lead to noticeable differences in infectivity for almost all the lyssavirus strains. The majority showed an increase in infectivity when cloned into pI.18 versus pcDNA3.1, whilst a couple (RABV-medoid, SHIBV-ev and IKOV-ev) showed a slight decrease. Both plasmid vectors are high copy number plasmids and use identical CMV immediate-early promoters for protein expression. Both plasmids have previously been used successfully as gene expression vectors in the creation of pseudotyped viruses [180,182].

It is currently unknown as to why pseudotype infectivity varies based on which of these two plasmids the glycoprotein is cloned into, given the similarities between them. The pI.18 plasmid contains a CMV intron A sequence between the CMV promoter and multiple-cloning site which may be affecting the efficiency of gene expression. Indeed, it has previously been shown that the presence of an intron A variant can improve transgene expression [220], however this does not explain why some lyssaviruses showed slightly reduced infectivity when cloned into this plasmid.

Interestingly, the VSV glycoprotein was also cloned into pI.18 and a substantial increase in infectivity over the pMD2.G plasmid was seen. The pMD2.G plasmid also contains an intron, β-globin, which can also increase transgene expression, which may indicate that expression varies based on the choice of intron [221]. Overall, this shows that choice of expression vector is an important consideration to make when developing a pseudotype platform and multiple vectors should be tested to identify the best for the virus of interest.

Protein blotting was attempted in order to test the theory that differences in glycoprotein expression levels may lead to improved pseudotype infectivity. However, whilst two different antibodies were tested at different concentrations and in both reducing and non-reducing conditions, neither were able to give off a signal. It is possible that neither antibody is suitable for protein blotting, however Rab50 has been used previously to detect immunoprecipitated RABV glycoprotein [222] whilst the 24-3F-10 antibody manufacturer claims the antibody has been validated for Western blotting.

Another possibility is that the total level of protein in the cell lysates may have been low. Whilst GAPDH gave off a signal under both reducing and non-reducing conditions, it was slightly faint at times despite the same cell lysates being used for each experiment. Protein quantification was not carried out prior to blotting as GAPDH was used as a positive control and the aim was to find appropriate blotting conditions before looking at differences in expression levels, which would require the same amount of protein to be loaded onto a gel.

Whilst the anti-RABV glycoprotein antibodies were unsuccessful, there are alternative ways to perform the blotting experiments that could potentially be used to look at differences in expression levels. One option would be to add a tag to the glycoproteins and then use an anti-tag antibody to detect protein expression. Another option would be to look at differences in expression for another glycoprotein such as VSV.

As two anti-RABV glycoprotein antibodies had failed to detect antigen, the decision was made to focus on collecting neutralisation data using the pseudotypes rather than continue investigating the differences between them, however this information would be valuable in providing a more complete picture as to why different plasmids lead to more infectious pseudotypes.

Differences in pseudotype infectivity could also be due to differences in the number of pseudotyped particles being produced. It is possible to increase the titre of pseudotypes by concentrating the particles by ultracentrifugation [223]. Determining an approximate pseudotype titre other than through measuring infectivity via the reporter gene would be a useful method of correlating infectivity to particle production. There are various methods of determining pseudotype titre, such as through ELISA measurement of pseudotype backbone or qRT-PCR of the pseudotype genome [169].

Pseudotype preparations are a heterogeneous mixture of defective interfering particles and functional pseudotypes and may also contain plasmid contamination from the transient transfection [224]. As such, pseudotype titres assessed on the preparations themselves may not reflect the functional titre of the infectious particles. Measuring the expression of the reporter gene through an infectivity assay, however, will estimate functional titre provided the level of expression is above the detection threshold [224]. As such, additional pseudotype titration was not performed.

Whilst switching to the use of the pI.18 plasmid instead of pcDNA3.1 allowed for the creation of infectious pseudotypes of most lyssaviruses, the RABV-GtB-ev

strain did not show any improvement in infectivity and consistently produced luminescence on par with the negative control ΔEnv pseudotype.

When the glycoprotein sequences were examined, it was noticed that all the lyssavirus strains had 14 conserved cysteine residues in their ectodomains. The exceptions to this were RABV-GtB-ev (which had 13) and EBLV-1 (which had 15). Cysteine residues are crucial for the formation of disulphide bonds, and thus proper protein folding. As the ectodomain of the glycoprotein protrudes from the surface membrane of the virus, it is responsible for interaction with cellular receptors and entry into cells. Appropriate folding of this region of the protein is therefore vitally important for virus infectivity.

Pairs of cysteine residues form disulphide bonds, bringing together two separate protein regions and stabilising protein folding. As the number and location of cysteine residues in the ectodomain are conserved across all lyssaviruses, regardless of sequence diversity, this implies that they are under strong selective pressure to enable the glycoproteins to fold appropriately. EBLV-1, the other lyssavirus with an unusual number of cysteine residues in the ectodomain, contained all 14 conserved cysteine residues plus an additional 15th residue at position 168, directly beside one of the conserved residues. This additional residue is therefore unlikely to interfere with protein folding.

RABV-GtB-ev however only has 13 cysteine residues in the ectodomain, which may prevent the formation of a disulphide bond leading to incorrect protein folding. This may then prevent the glycoprotein from interacting with the appropriate cellular receptors and entering cells.

In order to test this theory, SDM was performed on the RABV-GtB-ev strain to insert the missing cysteine residue and see if this would allow for the creation of infectious viral pseudotypes. Unfortunately, the mutated glycoprotein did not lead to a substantial increase in pseudotype infectivity, so the inability of this strain to infect BHK-21 cells may be unrelated to the cysteine residue count in the ectodomain.

It could be that this viral glycoprotein is unable to recognise the receptors present on these cells and can only infect cells from its original host, bats. Many of the other lyssaviruses present in this panel were also isolated from bats however, so if they are all using the same receptors it would seem unlikely that this would be the case. Bats are however, very diverse, and RABV-GtB-ev is the only lyssavirus isolated from a new world bat, *Desmodus rotundus*, whilst the other lyssaviruses in this panel are from old world bats such as *Eptesicus serotinus* and *Myotis daubentonii*, so it is possible that there are large enough differences between the receptors in these bats that they can affect lyssavirus infectivity.

It is also possible that RABV-GtB-ev pseudotypes had low levels of glycoprotein expressed on the surface. Pseudotypes form amorphous particles and lack the bullet-shaped morphology of lyssaviruses so will likely contain fewer glycoprotein molecules with no particular arrangement, in comparison to the uniformly-structured native virus particles [110,225]. If the structure of the RABV-GtB-ev glycoprotein is somewhat unstable due to the lacking cysteine residue in the ectodomain, it is possible that this could be overcome in the original infection by the structured expression of glycoprotein on the viral surface. One possible solution to this could be to switch the cytoplasmic domain of the RABV-GtB-ev glycoprotein with that of VSV, as this has previously been used to increase the pseudotype titre of RABV glycoproteins that displayed low infectivity [207,226]. As VSV is efficiently pseudotyped, this may lead to an increase in the amount of glycoprotein expressed on the pseudotype surface.

In summary, a panel of 12 lyssaviruses encompassing both *ex vivo* and lab-adapted strains has been developed, covering all 3 lyssavirus phylogroups, for the purpose of studying lyssavirus entry and neutralisation.

Chapter 4 – Neutralisation of a lyssavirus pseudotype panel using vaccinated serum

4.1 Introduction

RABV is the most widely circulating lyssavirus and is responsible for the majority of rabies infections worldwide, though the contribution of other lyssaviruses to rabies infection rates is unknown. Current vaccines against rabies encephalitis are based upon RABV and are an effective means of protecting against infection. The non-RABV lyssaviruses are also capable of causing rabies and are genetically distinct from RABV, therefore current vaccines may provide limited protection. Multiple studies have looked into cross-neutralisation of various lyssavirus species using both cell-based and animal-based infections [73,180,227,228]. All of these studies have shown reduced neutralisation against lyssaviruses, broadly correlating with genetic distance. Typically, neutralisation can be seen within lyssavirus phylogroups but not between them. The lack of protection against lyssaviruses other than RABV is not just a theoretical problem; MOKV infections have been identified in South African cats with a history of rabies vaccination [229].

Vaccination is currently the only method capable of protecting against and treating rabies infections. Neutralising antibody titre is used as a correlate for protection and a titre of 0.5 IU/ml is the minimum level required to indicate adequate vaccination. It is recommended that individuals at continual risk of exposure to rabies have their neutralising antibody titres monitored regularly and receive booster vaccinations if their titre falls below 0.5 IU/ml [3]. With antibody neutralisation being such a vital component of protection against rabies infection, it is important to have reliable assays capable of measuring antibody reactivity towards different strains of RABV and other lyssaviruses.

There are two main assays that are typically used to determine levels of rabies virus-neutralising antibodies. These are the fluorescent antibody virus neutralisation test (FAVN) and the rapid fluorescent focus inhibition test (RFFIT). Both of these tests involve treating a challenge virus with serum before

incubating with cells. The presence of virus is then detected using a specific anti-rabies antibody that is conjugated with a fluorescent marker such as fluorescein isothiocyanate (FITC). The main difference between the two methods is the final readout at the end: the FAVN classifies a well as positive for rabies infection if there are any fluorescent cells present, an all-or-nothing approach. The RFFIT, on the other hand, records the percentage of infected cells within a well [230]. The RFITT is considered to be the gold-standard of RABV neutralisation assays [231] however the FAVN is comparable in performance and was developed to help standardise the collection of results [232].

Pseudotype-based neutralisation assays have also been developed to help address some of the shortfalls with traditional neutralisation assays. As they use pseudotyped viruses rather than live virus strains they do not need to be performed in high-containment facilities and only require BSL-2 laboratories. Pseudotype neutralisation assays are also serum-sparing, enabling neutralisation assays to be performed with very small volumes of serum. As pseudotyped virus assays only contain the glycoprotein from the virus of interest, they also ensure that antibody responses measured are specific to the glycoprotein alone, rather than directed against other viral proteins [169]. The results from pseudotype-based neutralisation assays have previously been shown to correlate well with results obtained from the more traditional FAVN assay [180].

The work presented in this chapter aimed to establish a neutralisation assay to look at the cross-neutralisation provided by the current human rabies vaccine using the pseudotyped virus panel developed in Chapter 3.

4.2 Results

4.2.1. Neutralisation of the RABV-medoid strain with human and mouse serum

Initial work on setting up the neutralisation assay focused on the RABV-medoid strain, to determine that the pseudotypes were behaving as expected prior to expanding to look at all lyssavirus strains. As this work took place before the full

lyssavirus pseudotype panel was developed, the RABV-medoid and VSV pseudotypes were produced using the pcDNA3.1 and pMD2.G plasmids respectively, rather than pI.18.

Samples of human serum taken from an individual before and after rabies vaccination were diluted to 1:50 and 1:100 final concentrations and incubated with RABV-medoid pseudotypes for 1 hour prior to infecting BHK-21 cells. In order to ensure that any neutralisation seen was caused by antibodies specific to rabies virus, VSV pseudotypes were used as a negative control. Pseudotype infectivity was assessed 72 hrs later by measuring luminescence output. Data obtained was normalised relative to untreated pseudotypes (100% infectivity) and ΔEnv pseudotypes (0% infectivity).

The RABV-medoid pseudotypes showed a significant reduction in infectivity ($p<0.01$) when treated with serum taken post-rabies vaccination, whilst maintaining infectivity when treated with pre-vaccination serum. VSV pseudotypes showed no significant reduction in infectivity regardless of treatment with serum (Fig 4.1A).

RABV-medoid and VSV pseudotypes were also treated with mouse serum at a 1:100 final concentration. Mouse serum samples were taken from mice that had been vaccinated with an experimental rabies vaccine produced by our collaborators at ReiThera (Chapter 5). The neutralising antibody titres had previously been determined by the FAVN at ReiThera. Data from the mouse serum neutralisation test correlated well with the human serum neutralisation test, with decreasing levels of RABV-medoid neutralisation seen as antibody titres reduced. The VSV pseudotypes showed no significant reduction in infectivity regardless of antibody titre (Fig 4.1B).

4.2.2. Neutralisation of the full lyssavirus pseudotype panel

Once the full lyssavirus panel had been developed and optimised (Chapter 3) it was used in a neutralisation assay to assess the protective effects of the current human rabies vaccine against a broad spectrum of lyssaviruses.

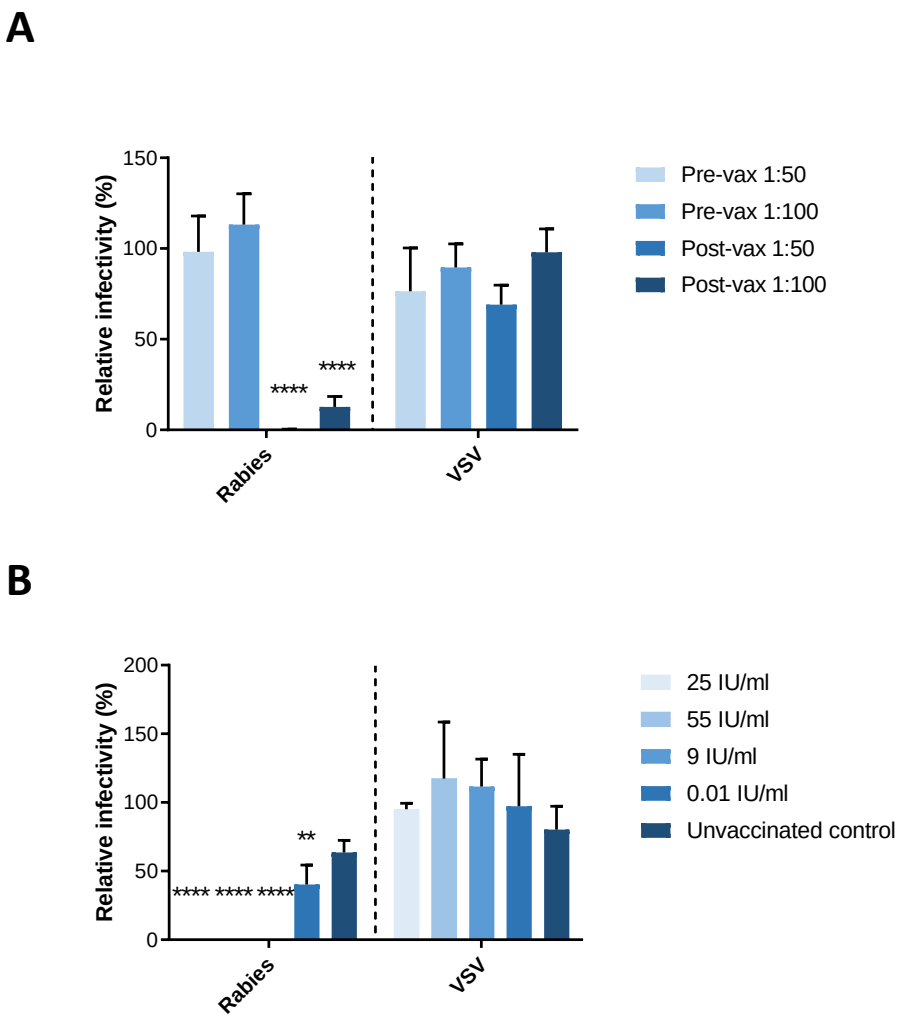


Figure 4.1 Neutralisation of RABV-medoid and VSV using human and mouse serum
Infectivity data of RABV-medoid and VSV pseudotypes neutralised with A) human and B) mouse serum, normalised relative to untreated pseudotype and ΔEnv controls. Human serum was tested at dilutions of 1:50 and 1:100 whilst mouse serum was tested at a 1:100 dilution. Wells were infected in triplicate and the human serum was tested in 2 independent experiments whilst the mouse serum was tested a single time due to limited amounts of sample. Significance was assessed by two-way ANOVA with Dunnett's correction and each sample was compared against the untreated pseudotype control (**$p < 0.005$; ****$p < 0.0001$). Error bars indicate standard deviation.

Human serum samples taken before and after rabies vaccination were diluted 1 in 50 and then underwent a 2-fold serial dilution. Serum samples were incubated with pseudotypes for 1 hr prior to addition to cells and infectivity measured 72 hrs later. Due to differences in pseudotype infectivity, not all pseudotypes could be measured at the same settings on the plate reader, with highly infectious pseudotypes (RABV-CVS, EBLV-1, EBLV-2, LBV-ev, LBV, SHIBV-ev, MOKV, WCBV, VSV) requiring a lower gain setting of 2000 and less infectious pseudotypes (RABV-medoid, RABV-GrD-ev, ABLV-ev, IKOV-ev) a gain of 3600. The data for each virus was normalised individually to an untreated control and the ΔEnv pseudotype.

The neutralisation assay showed that all phylogroup 1 lyssaviruses could be neutralised by vaccinated human serum whilst all of the phylogroup 2 and 3 lyssaviruses maintained infectivity (Fig 4.2). The RABV-medoid and RABV-GrD-ev pseudotypes showed a lot of variability in their infectivity when treated with pre-immune serum which was not seen in the other pseudotypes. The 1 in 2 serum dilution was not sufficient to produce full dose-response curves for the group 1 lyssaviruses, with several not returning to full infectivity at the lowest concentration of serum. The assay was therefore repeated using a 1 in 5 serum dilution (Fig 4.3).

The increased range of serum dilutions enabled full neutralisation curves to be calculated for the phylogroup 1 lyssavirus pseudotypes and for the IC_{50} values to be estimated (Table 4.1). The RABV-medoid strain was the most efficiently neutralised by the human serum, as shown by Fig 4.1 and the estimated IC_{50} values. EBLV-1 and EBLV-2 were the least efficiently neutralised as expected, being the most genetically distant phylogroup 1 lyssaviruses included in the panel. The RABV-CVS strain, however, was less efficiently neutralised than ABLV-ev, which was unexpected given that the human rabies vaccine is based off the RABV Flury low egg passage strain, which is closely related to RABV-CVS (Chapter 3, Fig 3.1).

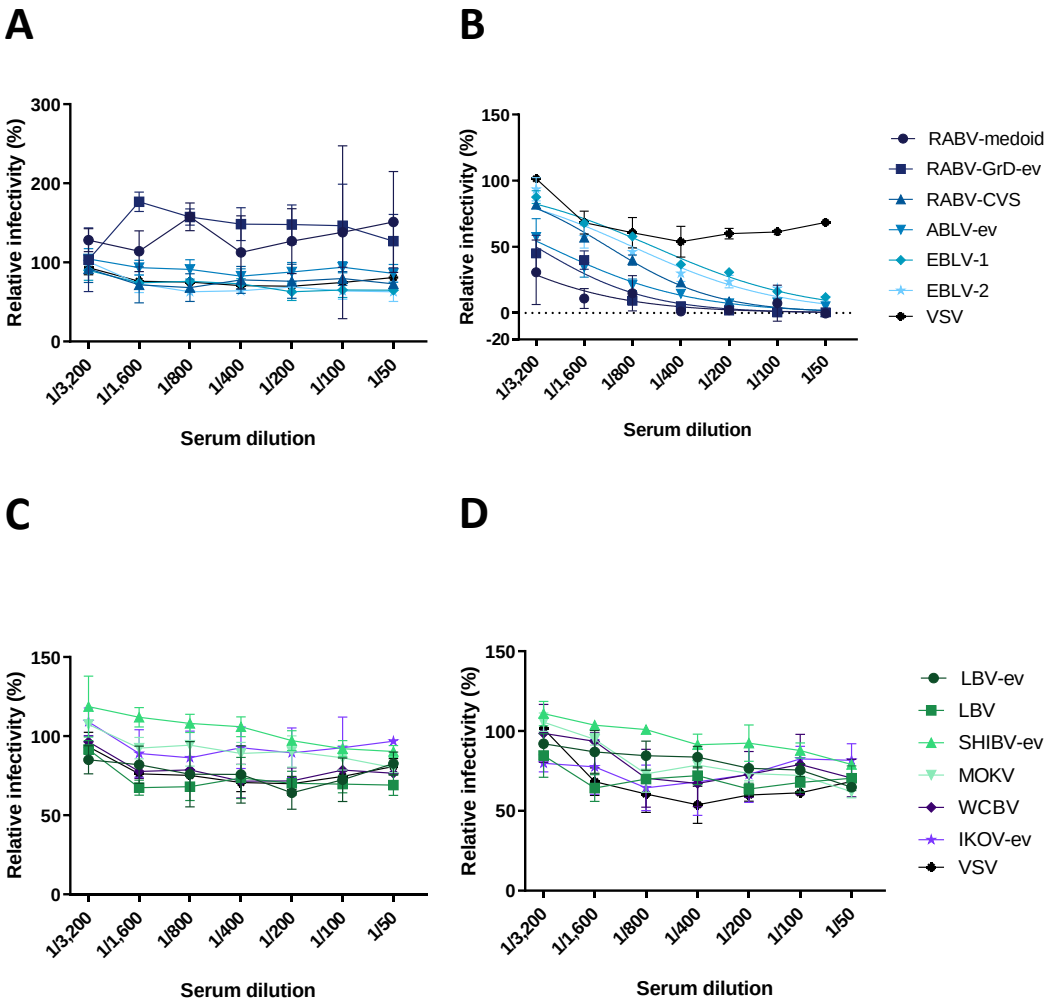


Figure 4.2 Neutralisation of lyssavirus pseudotypes with 2-fold serially diluted human serum

Phylogroup 1 (A, B) and phylogroup 2/3 (C, D) lyssavirus pseudotypes were treated with human serum taken pre- (A, C) and post- (B, D) rabies vaccination. Infectivity data was normalised relative to an untreated lyssavirus control and the ΔEnv control. Dose-response curves were calculated using Graphpad Prism for the phylogroup 1 lyssaviruses treated with vaccinated serum. Wells were infected in triplicate and the assay was repeated 3 times with the results presented being from a single representative experiment. Error bars indicate standard deviation.

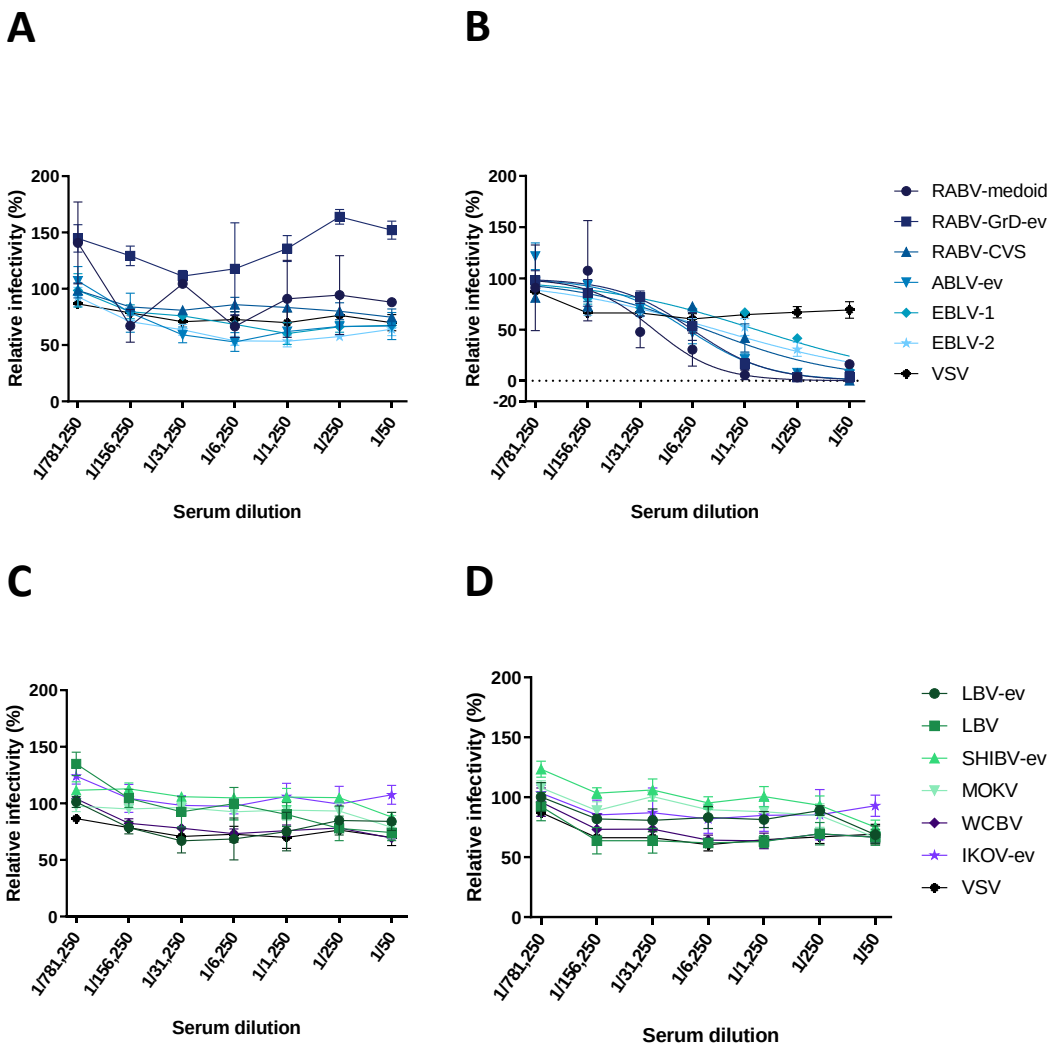


Figure 4.3 Neutralisation of lyssavirus pseudotypes with 5-fold serially diluted human serum

Phylogroup 1 (A, B) and phylogroup 2/3 (C, D) lyssavirus pseudotypes were treated with human serum taken pre- (A, C) and post- (B, D) rabies vaccination. Infectivity data was normalised relative to an untreated lyssavirus control and the ΔEnv control. Dose-response curves were calculated using Graphpad Prism for the phylogroup 1 lyssaviruses treated with vaccinated serum. Wells were infected in triplicate and the assay was only completed a single time. Error bars indicate standard deviation.

Table 4.1 Calculated IC_{50} values for group 1 lyssaviruses

Virus	IC_{50} (reciprocal dilution)	R^2
RABV-medoid	20,350	0.70
RABV-GrD-ev	6,309	0.95
RABV-CVS	4,167	0.84
ABLV-ev	7,225	0.93
EBLV-1	883	0.88
EBLV-2	2,958	0.89

4.2.3. Development of glycosylation mutants of phylogroup 1 lyssaviruses

The lyssavirus glycoprotein sequences were examined in an attempt to understand why the RABV-CVS strain would be less effectively neutralised than expected. Particular attention was paid to the antigenic regions, which are known to have an impact on viral neutralisation [140,142]. RABV-CVS was found to have a glycosylation site at position 204, close to antigenic site IIa. This glycosylation site was not present in the other group 1 lyssaviruses, though there was a glycosylation site at position 202 for all group 2 and 3 lyssaviruses (Fig 4.4). It was speculated that the glycosylation site at position 204 in RABV-CVS may have a role in masking the antigenic site from antibody recognition, thus reducing RABV-CVS neutralisation.

Site-directed mutagenesis was performed on the group 1 lyssaviruses RABV-GrD-ev, ABLV-ev, EBLV-1 and EBLV-2 in order to add an NKT glycosylation site at position 204. SDM was also carried out on RABV-CVS in order to remove the glycosylation site at position 204, by mutating the asparagine (N) residue to aspartic acid (D). The success of the SDM reaction was confirmed through Sanger sequencing.

Once all the glycosylation mutants had been developed, pseudotypes were created to see how the presence of the glycosylation site at position 204 affected infectivity. The highly infectious lyssaviruses RABV-CVS, EBLV-1 and EBLV-2 and their respective mutants were all read at a gain of 2000. It was found that the addition of the glycosylation site led to a large increase in infectivity for RABV-GrD-ev, ABLV-ev and EBLV-1. RABV-CVS showed comparable levels of infectivity regardless of the glycosylation site prevalence and EBLV-2 showed a slight, non-significant decrease in infectivity (Fig 4.5)

The glycosylation mutants were then used in a neutralisation assay. To streamline data collection, the highly infectious pseudotypes RABV-CVS, EBLV-1, EBLV-2 and their respective mutant strains were diluted in DMEM prior to addition to cells (RABV-CVS: 1:10, EBLV-1: 1:5, EBLV-2: 1:3). This enabled the infectivity of all pseudotypes to be read at the same optical gain of 3600.

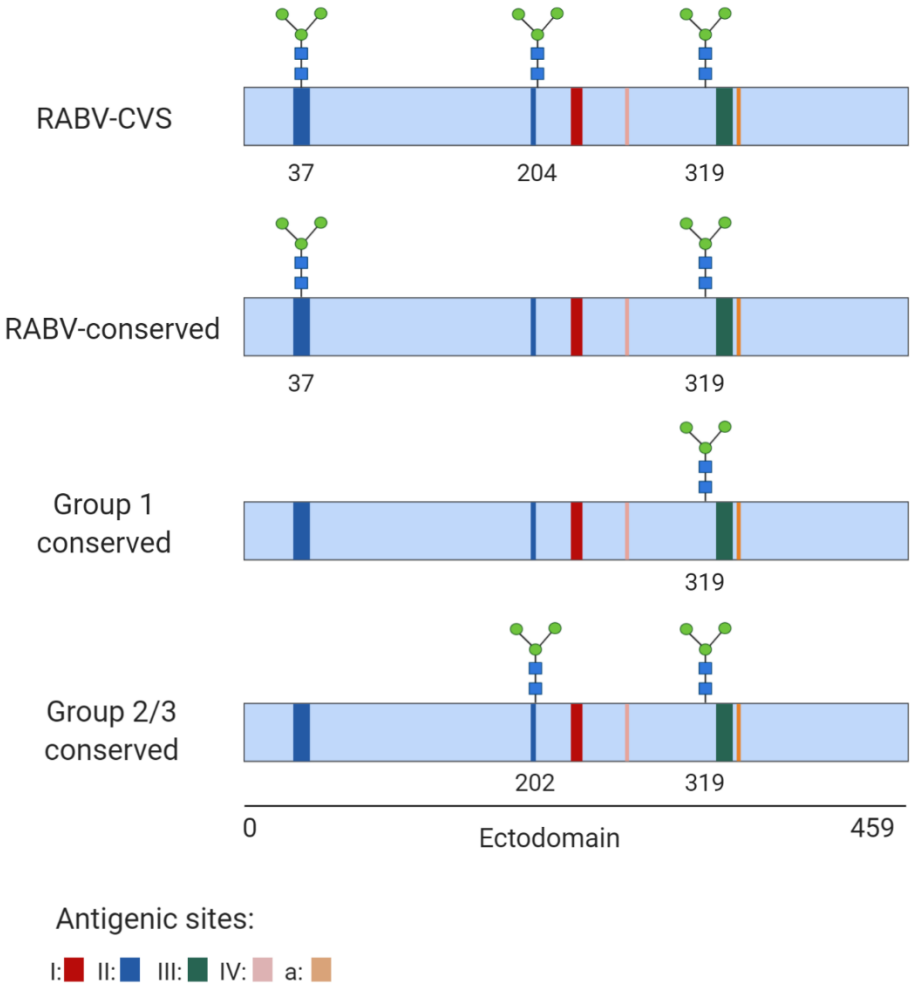


Figure 4.4 Locations of glycosylation sites within the ectodomain of the lyssavirus glycoprotein

A schematic showing the locations of conserved glycosylation sites and antigenic sites in the ectodomain of the lyssavirus glycoprotein. Numbering for amino acid residues follows the removal of the signal peptide.

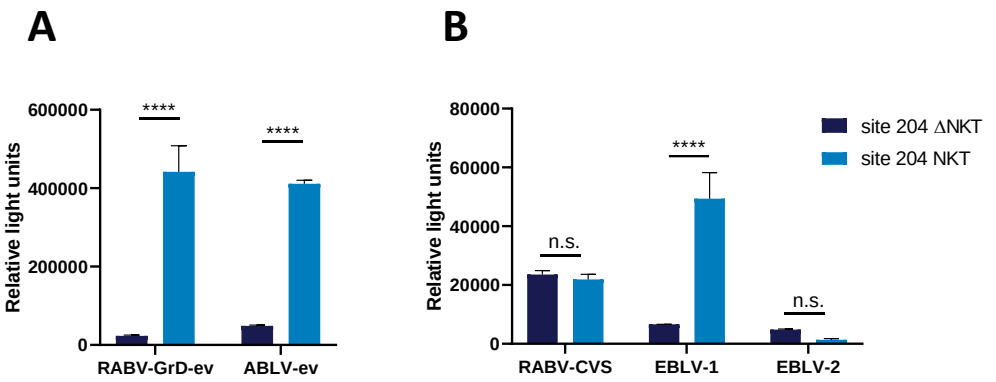


Figure 4.5 Infectivity of lyssavirus glycosylation mutants
Lyssavirus glycosylation mutants were used to infect BHK-21 cells and luminescence was measured in RLU at A) 3,600 and B) 2,000 optical gain. ΔEnv values were subtracted to remove background signal. Wells were infected in triplicate and the assay was repeated 3 times with the results presented being from a single representative experiment. Significance was assessed by two-way ANOVA with Sidak's correction (****$p < 0.0001$; n.s: not significant). Error bars indicate standard deviation.

As seen in figure 4.6, the lyssavirus strains containing a glycosylation site at position 204 had similar neutralisation profiles to the strains lacking a glycosylation site at this position. For RABV-CVS, the presence of a glycosylation site did appear to slightly increase resistance to neutralisation, and this was seen in two independent experiments. The presence of the glycosylation site seems to have little effect on the other lyssaviruses, though it is difficult to draw a solid conclusion due to the quality of the data.

4.3 Discussion

Pseudotype neutralisation assays are sensitive and specific assays that can be used to assess the immune response to viral infection [169]. Results from these assays are comparable to those obtained using live viruses [180,197]. In this chapter the lyssavirus pseudotype panel was neutralised with vaccinated human serum to investigate the differing levels of protection provided against various lyssavirus strains.

Lyssavirus neutralisation with human serum led to broadly expected results, with no neutralisation seen for the phylogroup 2 and 3 lyssaviruses or for the VSV negative control. Neutralisation efficiency varied amongst the phylogroup 1 lyssaviruses, which confirms results seen in previous studies [73,180,227,228].

Viral membrane proteins are exposed to high selective pressures due to their location on the surface of the virus particle and therefore being a primary target of the host immune response. This can lead to high levels of genetic variability and this is also seen within the lyssavirus glycoprotein. As shown in table 4.2, there can be as high as 30% amino acid variability within a phylogroup and approximately 50% variation across phylogroups. WCBV and IKOV are tentatively grouped as part of a third phylogroup but share only 48% amino acid identity. With such genetic variation, it is unsurprising that there is little cross-neutralisation between phylogroups. Other studies have shown that some antibodies are capable of neutralising lyssaviruses from both groups 1 and 2, but typically only to a limited extent [69,227].

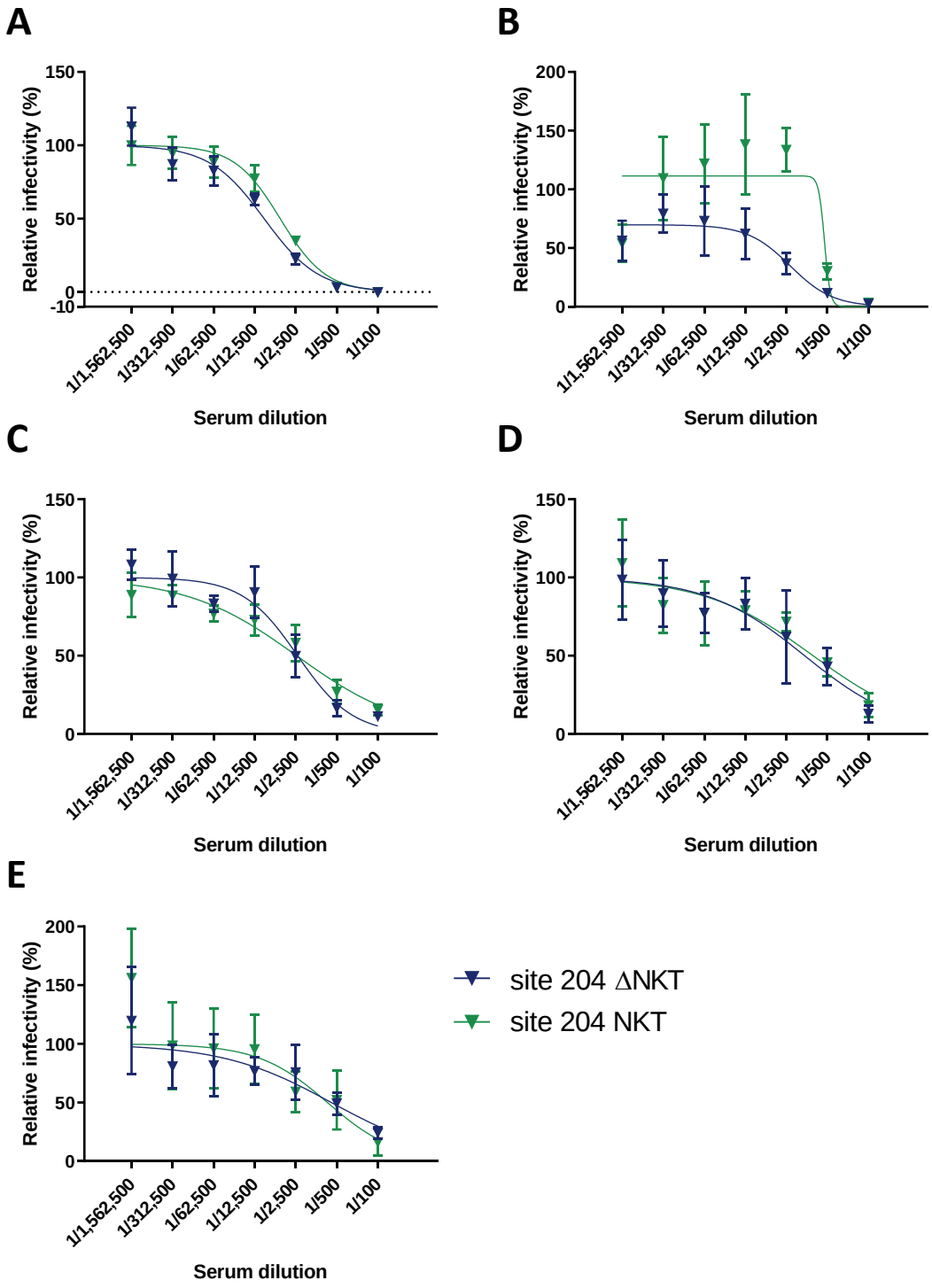


Figure 4.6 Neutralisation of lyssavirus glycosylation mutants with human serum
Lyssavirus pseudotypes A) RABV-CVS, B) RABV-GrD-ev, C) ABLV-ev, D) EBLV-1 and E) EBLV-2 were modified to either express (NKT) or lack (ΔNKT) a glycosylation site at position 204. Infectivity data was normalised relative to untreated lyssavirus controls and the ΔEnv control. Dose-response curves were calculated using Graphpad Prism. Wells were infected in triplicate and results were aggregated from two independent experiments for all lyssaviruses except RABV-GrD-ev (B) and ABLV-ev (C). Error bars indicate standard deviation.

Table 4.2 Amino acid percentage identities of the lyssavirus glycoprotein

Percentage identities are shown. The multiple sequence alignment and percentage identity matrix were produced using MUSCLE. Cells are shaded with increasing intensity of green as the percentage identity increases.

	Group 1							Group 2				Group 3	
	1: RABV Medoid	2: RABV CVS	3: RABV GrD-ev	4: RABV GtB-ev	5: ABLV-ev	6: EBLV-1	7: EBLV-2	8: LBV-ev	9: LBV	10: SHIBV-ev	11: MOKV	12: WCBV	13: IKOV-ev
1: RABV-Medoid	100												
2: RABVCVS	93.13	100											
3: RABV-GrD-ev	95.42	91.03	100										
4: RABV-GtB-ev	86.45	85.11	85.31	100									
5: ABLV-ev	78.63	76.72	77.1	76.15	100								
6: EBLV-1	72.03	70.5	71.65	70.5	75.95	100							
7: EBLV-2	76.63	74.33	75.29	73.18	79.96	79.96	100						
8: LBV-ev	58.24	57.28	58.24	56.7	58.81	59.04	55.96	100					
9: LBV	58.62	57.09	58.05	56.7	58.81	58.08	57.31	80.27	100				
10: SHIBV-ev	59.39	58.81	60.15	58.81	59.58	60	58.27	78.35	79.12	100			
11: MOKV	59	57.47	58.43	58.24	57.85	58.27	56.92	76.82	73.95	72.03	100		
12: WCBV	50.96	50.77	50.57	48.47	51.72	50.96	50.96	53.45	53.64	52.87	51.92	100	
13: IKOV-ev	48.18	47.02	47.79	47.02	47.6	48.17	48.75	49.71	46.83	48.94	48.75	48.09	100

With the phylogroup 1 neutralisation data shown in Fig 4.3, the main surprise was how resistant the RABV-CVS strain was to neutralisation, given how closely related it is to the rabies virus vaccine strain. The glycosylation site at position 204 was identified as potentially being involved, given its close proximity to antigenic site IIa and that it was not present in the other group 1 lyssaviruses.

Glycosylation sites are known to impact on virus neutralisation and are capable of masking antigenic sites from antibody detection. It has previously been shown that the removal of glycosylation sites from HIV-1 gp120 can affect the neutralisation sensitivity of the virus [130]. There is also evidence that adding additional glycosylation sites to the haemagglutinin molecule of influenza viruses can increase viral resistance to antibody recognition [233]. These viral proteins have heavily glycosylated regions which are thought to help the virus evade immune detection. The lyssavirus glycoprotein is sparsely glycosylated in comparison, with only 1 conserved glycosylation site seen on all lyssaviruses at position 319. This glycosylation site is likely important in lyssavirus infectivity and previous studies on RABV glycosylation have indicated that it is efficiently glycosylated [129], has a role in enabling the glycoprotein to be transported to the cell surface and is required for the secretion of soluble RABV glycoprotein [135].

The only other conserved glycosylation site is at position 202 in the phylogroup 2 and 3 lyssaviruses. This glycosylation site was not observed on any of the group 1 viruses and there have been no previous studies into its role in the glycoprotein, though given its conserved nature it may be involved in glycoprotein folding or transportation to the cell surface.

The glycosylation site on RABV-CVS at position 204 is close to antigenic site IIa and so may have led to increased resistance to neutralisation, by interfering with antibody binding. Antigenic site II has previously been shown to be important in RABV neutralisation [139,140] so it is feasible that the presence of a large sugar molecule could be having an effect on antibody binding.

SDM was performed on all group 1 lyssaviruses, except RABV-medoid, so that each lyssavirus had 2 versions: NKT+ and NKT- at position 204. The addition of the glycosylation site had an effect on the infectivity of all the lyssaviruses, with RABV-CVS and EBLV-2 decreasing in infectivity, and the others all showing a large increase in infectivity. Glycosylation sites can have an influence on viral infectivity by enhancing or interfering with receptor binding [128], so it may be that slight differences in lyssavirus glycoprotein structures led to different effects on pseudotype infectivity.

Neutralisation of the glycosylation mutants showed that the mutants behaved in a similar manner to the non-mutated strains with the neutralisation curves strongly resembling the original lyssavirus. There did appear to be a slight increase in resistance to neutralisation for RABV-CVS as the curves were slightly shifted towards higher serum concentrations. The change was not large enough to explain fully why RABV-CVS was more resistant to neutralisation than ABLV-ev but did indicate that glycosylation may have an effect on antibody resistance. This did not appear to be seen in the other virus strains, which may imply that this was a RABV-specific effect.

Unfortunately, the data quality for some of the other lyssaviruses was poor, with high variation between replicates. This was particularly noticeable for RABV-GrD-ev and was exacerbated by the fact that there was only data from one experiment available. As one of the less infectious lyssavirus pseudotypes, RABV-GrD-ev was very sensitive to issues with pseudotype production, leading to minimal infectivity. Whilst steps were taken to increase infectivity, by defrosting fresh cells and creating a fresh batch of PEI, there was only enough time to attempt one neutralisation assay with the fresh batch of pseudotype. Particularly high infectivity values were seen for the RABV-GrD-ev-NKT mutant when treated with serum, higher than the untreated control. This prevented useful neutralisation curves from being established. Whilst ABLV-ev also suffered from low infectivity in the initial experiment, the data from the following experiment was sufficient to form reasonable neutralisation curves.

Whilst it is difficult to form a solid conclusion on the impact of the glycosylation site at position 204 on neutralisation, the RABV-CVS data suggests that the effect is likely to be slight.

In conclusion, in this chapter neutralisation assays were set up and the results confirmed previous work showing a lack of cross-neutralisation between lyssavirus phylogroups. Glycosylation of a site close to antigenic site IIa was also investigated for its effects on lyssavirus neutralisation and appeared to have a limited effect that may be localised to RABV-CVS alone, though the results are still inconclusive at this time.

Chapter 5 – Investigation into the neutralisation profile of a novel rabies vaccine compared to the current human vaccine

5.1 Introduction

Vaccines providing protection against rabies have been around since the late 1800s when Louis Pasteur developed the first rabies vaccine by passaging the virus through rabbits to attenuate it and drying out the nerve tissue to decrease virulence [45]. Rabies vaccines have been developed a great deal in the 100 years since the first vaccine was produced and nerve tissue vaccines are no longer recommended due to the high likelihood of adverse side-effects [234,235]. Currently recommended vaccines use attenuated rabies virus strains grown in cell culture or in embryonated eggs [234].

Whilst the current rabies vaccines are very effective and PEP failure is rare when guidelines are followed appropriately [43], multiple doses are required to be administered on different days, with a three-dose regimen being recommended for pre-exposure prophylaxis and a four or five-dose regimen for post-exposure prophylaxis [3,234]. Combined with the costs of receiving RIG, it can be an expensive treatment even assuming adequate supplies are available in the developing nations where rabies prevalence is highest [236]. Whilst there is a clear need for more immunogenic rabies vaccines to streamline the current treatment schedule, potential novel vaccine candidates have high barriers to overcome as the high mortality rate of rabies means there is no room for error and candidates cannot risk being less effective than currently available options.

Various different strategies for developing novel rabies vaccines are being currently explored including mRNA vaccines [50], baculovirus-based vectors [237], improved adjuvants [59] and genetically modified rabies virus strains [238]. One interesting strategy involves adenovirus-based vaccine vectors.

Adenoviruses are capable of inducing host immune responses upon infecting cells and can be easily modified to express foreign antigens [239]. They are highly immunogenic and can be replication-competent or incompetent

depending on whether the *E1* gene is present. Replication-incompetent adenovirus vaccines could potentially be less immunogenic than replication-competent vectors, however vectors that are replication-competent will compete with the antigenic transgene for immune response induction, as all the vector's genes will be expressed [240].

Whilst adenoviruses are a good candidate for a vaccine vector, they are not without issues. Adenoviruses are common viruses that cause cold-like symptoms in individuals and the majority of populations will display pre-existing immunity to common adenovirus serotypes [239]. One way around this is to use rare adenovirus serotypes or non-human adenovirus strains. Chimp adenoviruses (ChAd) are closely related to human adenoviruses and have low seroprevalence in humans of 1-4% in the US increasing to approximately 20% in sub-Saharan Africa [241] and Brazil [242].

Our collaborators in ReiThera developed a novel chimp adenovirus-based rabies vaccine using the RABV-medoid glycoprotein, with the goal of creating a more immunogenic vaccine than current rabies vaccines. This could potentially mean that fewer doses are required to provide protection against rabies. The RABV-medoid glycoprotein was used as the surface antigen in the hope of increasing cross-neutralisation against a greater breadth of lyssaviruses. This vaccine has been trialled in animal studies and serum samples from vaccinated non-human primates were provided for use in pseudotype neutralisation assays to compare the responses between the novel vaccine and a current human rabies vaccine.

5.2 Results

5.2.1. Non-human primate vaccination

In brief, the non-human primate (NHP) vaccination study by ReiThera was performed as follows. Cynomolgus monkeys (*Macaca fascicularis*) were divided into three groups of five and received three different rabies vaccination schedules. Group 1 was vaccinated with the novel ChAd155-RG vaccine once in

week 0 and received a single boost in week 48. Group 2 was vaccinated with the novel ChAd155-RG vaccine once in week 0, received a boost of the Rabipur human rabies vaccine in week 24 and received a further boost with the ChAd155-RG vaccine in week 48. Group 3 was vaccinated with the Rabipur vaccine following the standard schedule, with three doses given on days 1, 7 and 21 of the study. Animals in group 3 were followed up for 6 months, whilst those in groups 1 and 2 were followed up for 1 year post-vaccination (Fig 5.1). Serum samples were taken at regular intervals, though only a selection of these were provided for neutralisation assays (Table 5.1).

A total of 35 serum samples were available for use in neutralisation assays. Due to the size of the lyssavirus panel, it was unfeasible to generate neutralisation curves for all serum samples against all virus strains, so it was decided to focus on samples from groups 1 and 3 alone. Samples from group 2 were not included because this group of animals received both the ChAd155-RG and the Rabipur vaccine, which would make the effect of the vaccines more difficult to analyse. Samples taken at day 0 and week 5 post-vaccination were therefore used as they would show how the individuals responded to the two vaccines at the same time point, so should be the most comparable. There were only 3 samples available for group 1 at the week 5 time point, and 4 available for group 3. A selection of lyssaviruses from the pseudotype panel were chosen for comparison of neutralisation profiles: RABV-CVS, EBLV-1, EBLV-2 and MOKV. RABV-CVS was chosen as a positive control, since it is a lab-adapted strain that is commonly used for neutralisation assays. EBLV-1 and EBLV-2 were both selected as they are phylogroup 1 lyssaviruses that can both be neutralised, but to a lesser extent than RABV and because these pseudotypes had a higher yield than ABLV-ev, making them easier to work with. MOKV was chosen as a representative phylogroup 2 lyssavirus because it is known that MOKV can infect vaccinated animals [229] and because a limited amount of cross-neutralisation has been previously seen with this lyssavirus [69,227].

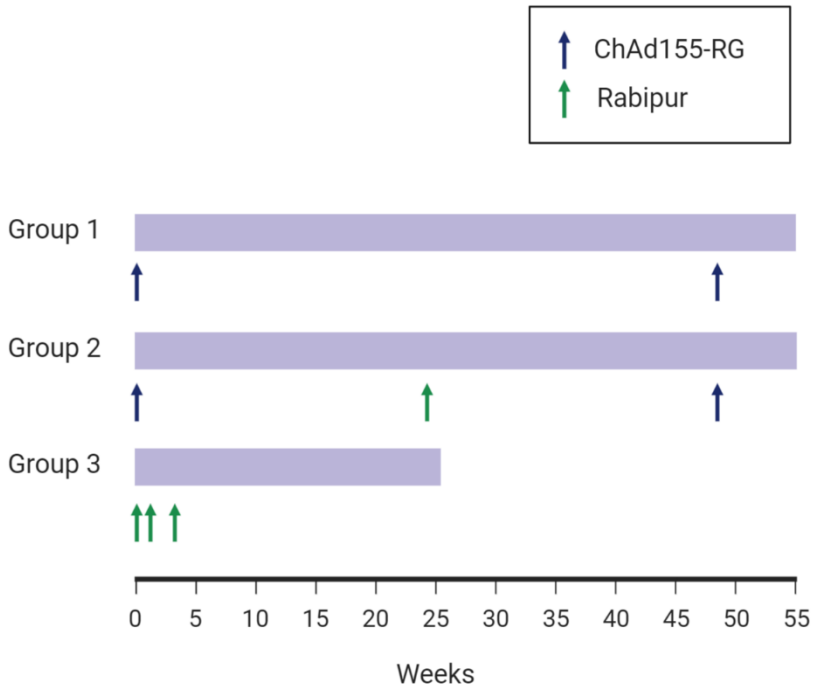


Figure 5.1 Timeline of non-human primate vaccination schedule
A schematic representing the vaccination schedule for the 3 groups of non-human primates. Bars indicate the period of time each group was monitored for in the study, with arrows indicating when vaccines were administered.

Table 5.1 Serum samples available for neutralisation assays

Serum samples available from individual animals at various time points in the study. Individual animals were designated with a number between 1 and 15. Animals were vaccinated as per the schedule shown in figure 5.1.

	Day 0	Week 3	Week 5	Week 27	Week 50
Group 1	1, 2, 3, 4, 5		1, 2, 4		1, 2, 3, 4, 5
Group 2	6, 7, 8, 9, 10		7, 9	6, 7, 8, 9, 10	
Group 3	11, 12, 13, 14, 15	11	12, 13, 14, 15		

5.2.2. Neutralisation comparison between ChAd155-RG and Rabipur vaccinated serum

Lyssavirus pseudotypes were produced as previously described (Chapter 3), however rather than using them straight away for assays they were frozen in aliquots. Whilst fresh preparations of pseudotypes had to be used within 2 weeks of collection before infectivity would drop, the frozen aliquots were still viable up to 1 month after collection. All pseudotypes were diluted in DMEM after being defrosted, both to allow results to be read at an optical gain of 3600 and to ensure there was sufficient volume of pseudotyped virus available for the neutralisation assays.

Serum samples were heat inactivated and diluted 1 in 100 with PBS, then serially diluted 1 in 5. Serum samples were incubated with pseudotypes for 1 hr prior to addition to cells and infectivity measured 72 hrs later. The data was then normalised relative to an untreated control and the ΔEnv control.

The majority of serum samples taken at day 0 did not show any neutralisation of any viruses (Fig 5.2) however serum from one animal in group 3 (Cy15) did show neutralisation at the day 0 time point (Fig 5.3). This was particularly noticeable for EBLV-1 and EBLV-2, though there is a drop in infectivity for RABV-CVS at the highest serum concentration as well. This neutralisation was not seen for VSV or MOKV and appears to be specific to group 1 lyssaviruses. Several aliquots were taken from the original serum stock and heat inactivated, however the neutralisation at day 0 remained. Results from this animal were therefore excluded from analysis.

The week 5 serum from all animals showed neutralisation of RABV-CVS, EBLV-1 and EBLV-2, with no neutralisation seen for MOKV or the VSV negative control (Fig 5.4). The ChAd155-RG and Rabipur serum showed comparable neutralisation curves against RABV-CVS however there was a distinct difference for EBLV-1 and EBLV-2, with the ChAd155-RG serum showing slightly more efficient neutralisation. When the IC_{50} figures were compared, a significant

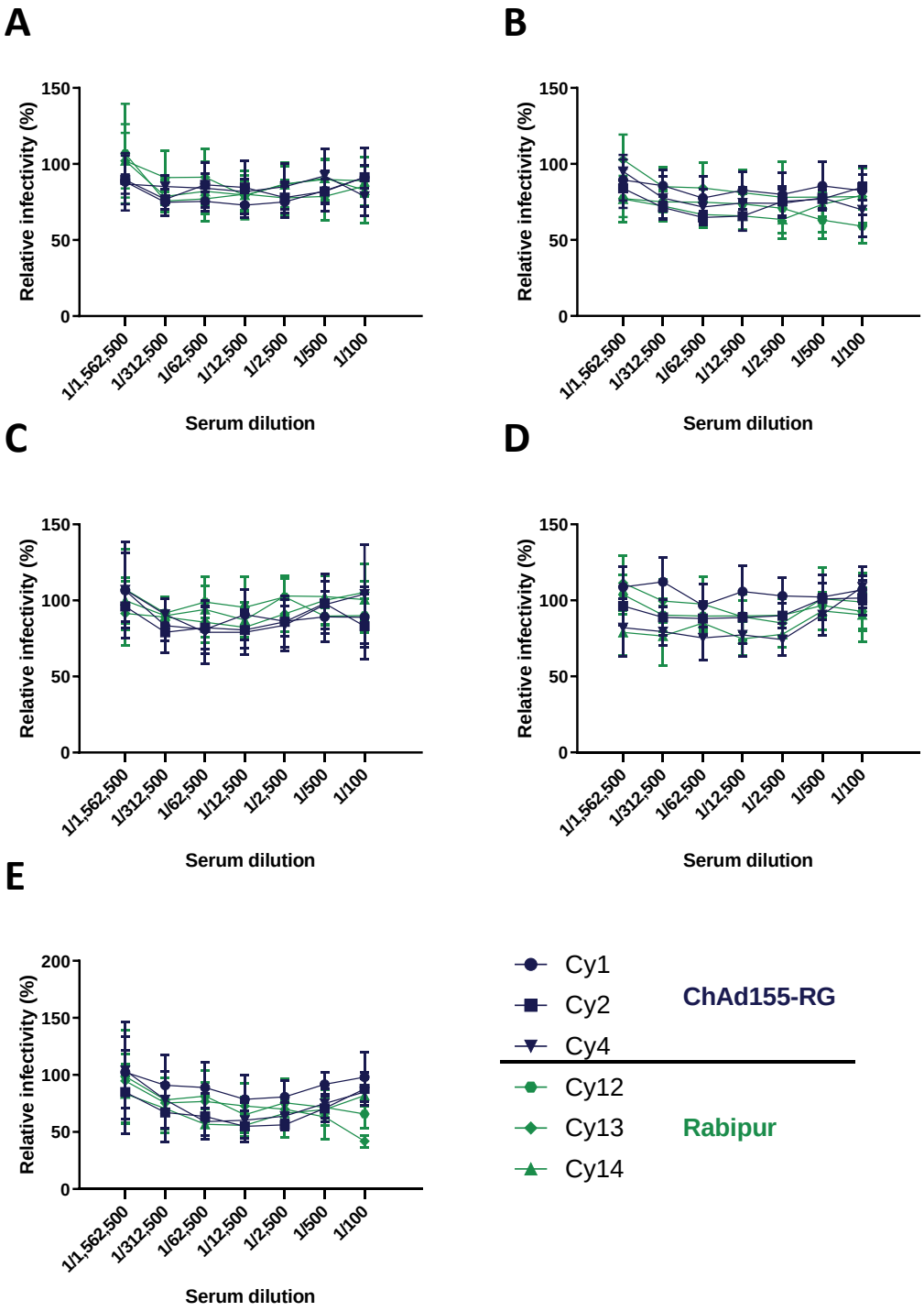


Figure 5.2 Lyssavirus neutralisation using pre-vaccinated serum
Serum was taken from NHPs prior to vaccination and tested for neutralisation capabilities against A) RABV-CVS, B) EBLV-1, C) EBLV-2, D) MOKV and E) VSV. Infectivity data was normalised relative to an untreated lyssavirus control and the ΔEnv control. Wells were infected in triplicate and results are aggregated from two independent experiments. Error bars indicate standard deviation.

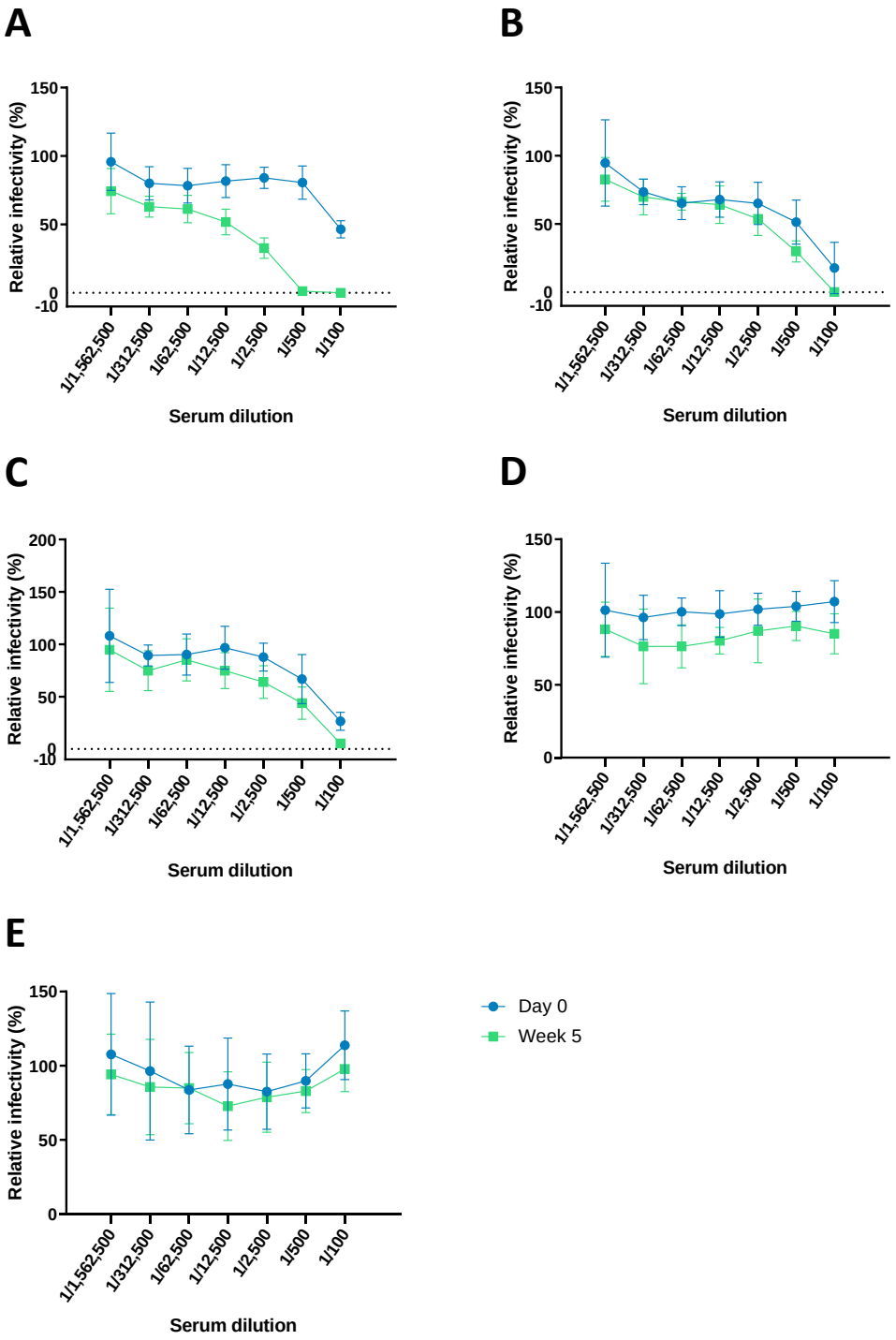


Figure 5.3 Lyssavirus neutralisation comparison between pre-vaccinated and post-vaccinated serum for animal Cy15

Serum was taken from the animal Cy15 prior to vaccination and on week 5 post-vaccination with the Rabipur human rabies vaccine and tested for neutralisation capabilities against the lyssaviruses A) RABV-CVS, B) EBLV-1, C) EBLV-2, D) MOKV and E) VSV negative control. Infectivity data was normalised relative to an untreated control and the ΔEnv control. Wells were infected in triplicate and results are aggregated from two independent experiments. Error bars indicate standard deviation.

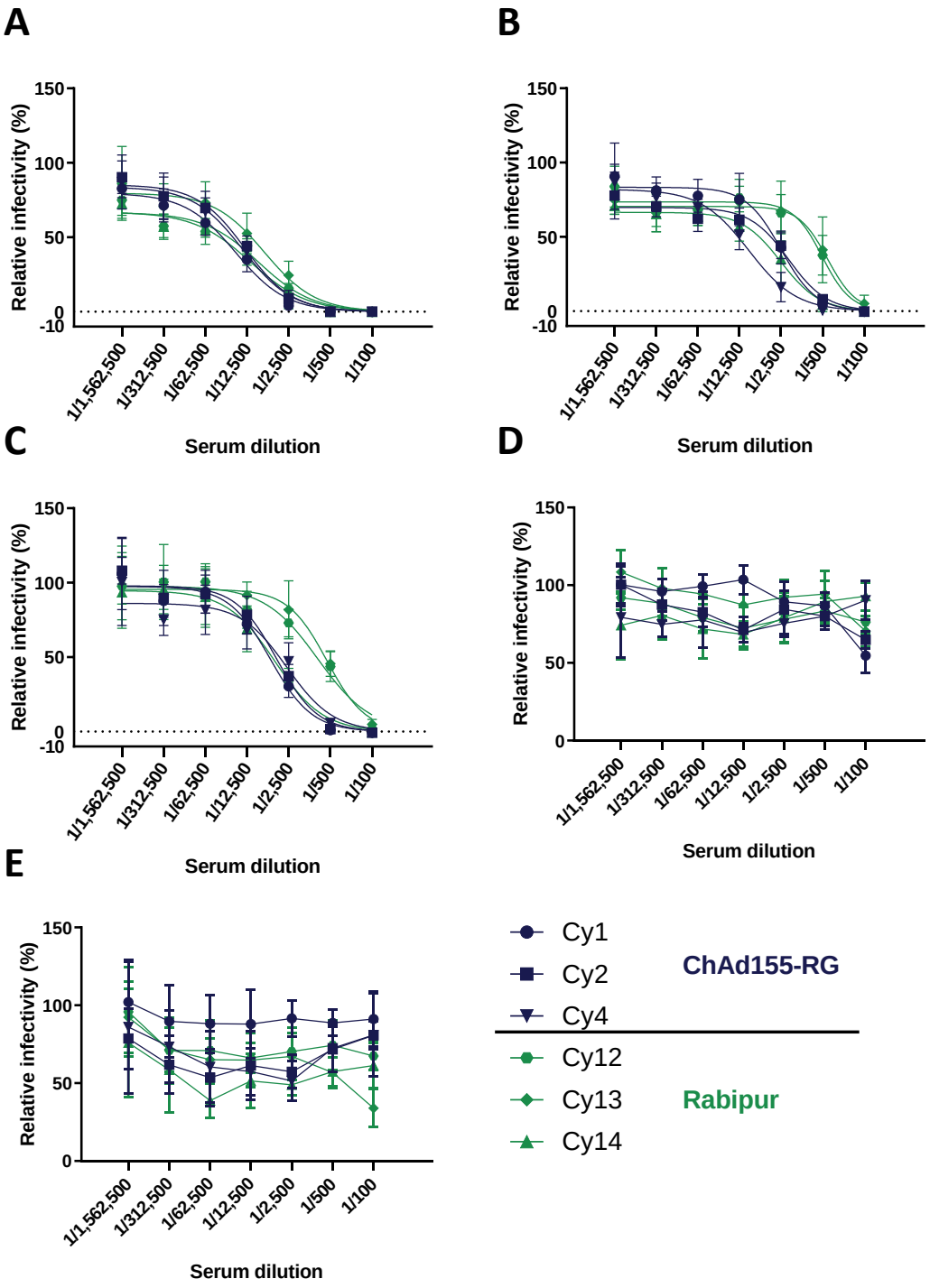


Figure 5.4 Lyssavirus neutralisation by serum from NHPs vaccinated with novel vaccine ChAd155-RG or Rabipur

Serum was taken from NHPs 5 weeks post-vaccination and tested for neutralisation against the viruses A) RABV-CVS, B) EBLV-1, C) EBLV-2, D) MOKV and E) VSV. Infectivity data was normalised relative to an untreated lyssavirus control and the ΔEnv negative control. Dose-response curves were calculated using GraphPad Prism. Wells were infected in triplicate and results are aggregated from two independent experiments. Error bars indicate standard deviation.

difference can be seen between ChAd155-RG and Rabipur for EBLV-1, though the difference is non-significant for EBLV-2 (Fig 5.5).

There were only 3 samples available for group 1 at the week 5 time point however samples from the 2 missing animals were available at week 50, taken after the animals had received a booster ChAd155-RG vaccination. These samples were therefore used in a brief pilot study to see if the booster vaccination led to an improvement in immune response. These 2 samples were tested once against the same lyssaviruses as the other samples. As shown in figure 5.6, these samples were capable of neutralising all the lyssaviruses including MOKV, but remained negative against VSV, showing the response was specific to lyssaviruses. The neutralisation response against RABV-CVS, EBLV-1 and EBLV-2 was also stronger than that seen at week 5, with much lower IC_{50} values (Fig 5.7).

5.3 Discussion

In this chapter, pseudotyped virus neutralisation assays were used to compare the neutralisation profiles of two different vaccines using serum taken from NHPs in a vaccination study. The aim was to identify if the two vaccines produced comparable immune responses and whether there were any differences in cross-neutralisation ability.

As expected, serum taken pre-vaccination did not neutralise any of the lyssavirus strains tested, showing the animals were in a naïve state at the start of the experiment. The one exception to this was an animal from group 3, Cy15, which showed a particularly strong neutralisation response to EBLV-1 and EBLV-2 and was capable of causing a low amount of neutralisation of CVS-11 at high serum concentrations (Fig 5.3).

This neutralisation was seen even when different aliquots of the original sample were taken and heat inactivated, indicating that it was something present in the original stock. Potentially this stock could have been contaminated with some

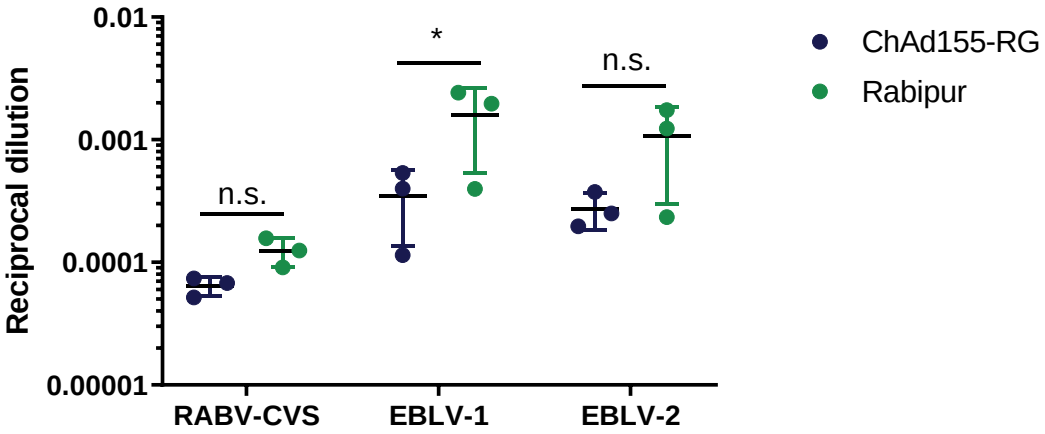


Figure 5.5 IC_{50} serum neutralisation values

NHPs were vaccinated with either ChAd155-RG or Rabipur and serum collected 5 weeks post-vaccination. IC_{50} values were calculated from dose-response curves using GraphPad Prism. Significance was assessed by two-way ANOVA with Sidak's correction ($^*p < 0.05$). Error bars indicate standard deviation.

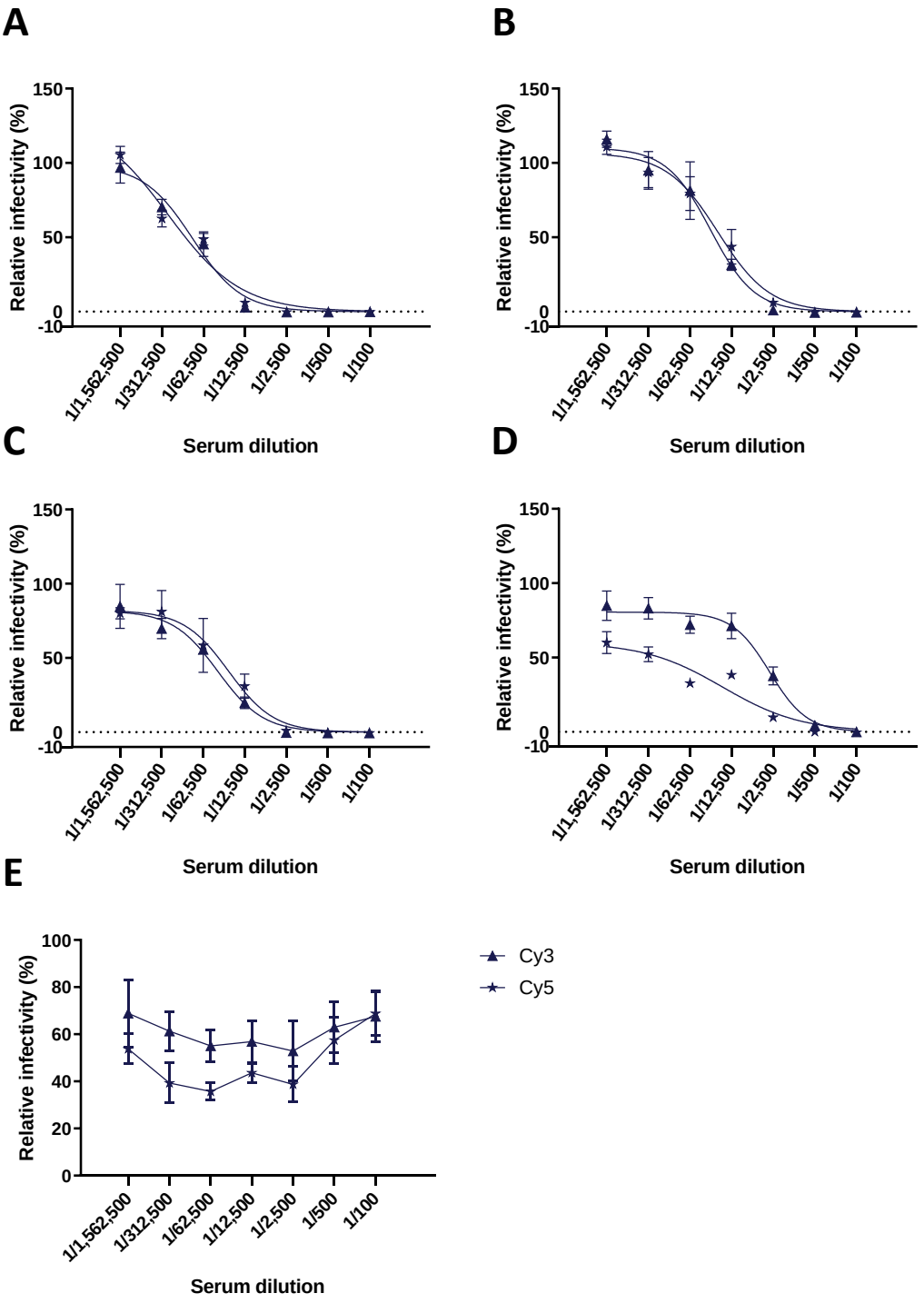


Figure 5.6 Lyssavirus neutralisation by ChAd155-RG vaccinated serum following a boost at week 48

Serum was taken from NHPs during week 50 of the experiment, 2 weeks after a boost vaccination was administered. Neutralisation properties were tested against the viruses A) RABV-CVS, B) EBLV-1, C) EBLV-2, D) MOKV and E) VSV. Infectivity data was normalised relative to an untreated lyssavirus control and the ΔEnv negative control. Dose-response curves were calculated using GraphPad Prism. Wells were infected in triplicate and the assay was carried out a single time. Error bars indicate standard deviation.

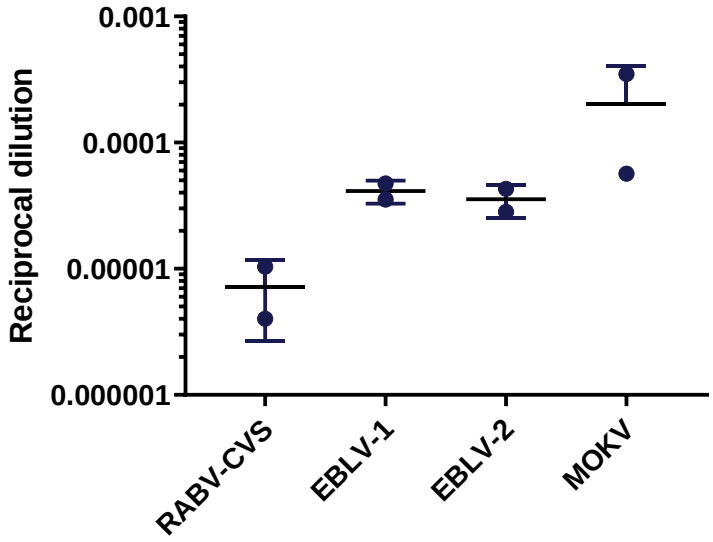


Figure 5.7 IC_{50} serum neutralisation values for post-boost vaccinated serum

NHPs were vaccinated with two doses of ChAd155-RG administered in weeks 1 and 48. Serum was collected in week 50. IC50 values were calculated from dose-response curves using GraphPad Prism. Error bars indicate standard deviation.

post-vaccination serum, however looking at the curves this seems unlikely. Had the stock been contaminated accidentally with a small amount of post-vaccination serum, this would have been heavily diluted by the serum already present, thus leading to a reduced neutralisation response, potentially similar to that seen against RABV-CVS. However, EBLV-1 and EBLV-2 showed responses on par with the post-vaccination serum which would imply that it was not caused by a diluted contaminant.

The fact that the response is mostly specific to these two lyssaviruses and is reduced against RABV-CVS is also interesting as the other post-vaccinated serum samples are capable of neutralising RABV-CVS more efficiently than EBLV-1 or EBLV-2. Taken together, this suggests there is some factor present in the original serum that is capable of neutralising group 1 lyssaviruses that was not removed by heat inactivation.

The NHPs were purpose-bred and housed in a dedicated NHP unit and were monitored for disease throughout the study, therefore it is unlikely that any animals were exposed to any lyssavirus prior to the start of the study.

Serum contains many different proteins that could potentially interfere with the neutralisation assay. Complement proteins are a part of the innate immune system that are capable of activating through three different pathways and ultimately leading to viral or cellular lysis [243]. Complement proteins are also capable of enhancing antibody responses to viruses by opsonising the surface of the virus. As the complement system plays a major role in combating viral infection, serum used in neutralisation studies is routinely heat-inactivated in order to disrupt the complement proteins and eliminate their antiviral effects [243].

Other non-antibody proteins present in serum that could interfere in virus infection include cytokines, chemokines and soluble lectins. RANTES, MIP-1α and MIP-1β are chemokines that have been previously shown to be capable of reducing HIV-1 infectivity [244], whilst soluble lectins such as mannose-binding

lectin have been shown to be capable of neutralising hepatitis C pseudotypes through binding to the hepatitis C E1/E2 glycoprotein [245,246].

Future work to uncover the provenance of the neutralisation ability displayed by Cy15 prior to vaccination could involve isolating total IgG from the serum to determine whether this pre-vaccination neutralisation is antibody-mediated or is caused by one of the other components of serum.

The neutralisation profiles of ChAd155-RG and Rabipur were comparable for RABV-CVS however there was a distinct separation between the curves for EBLV-1 and EBLV-2, with the ChAd155-RG serum neutralising these lyssaviruses at slightly lower serum concentrations than the Rabipur. This is reflected in the IC_{50} values, with the Rabipur serum samples showing slightly increased IC_{50} values.

There were only 3 samples available for each vaccinated group at the week 5 time point however, and whilst the ChAd155-RG samples clustered together giving very comparable curves and IC_{50} values, one of the Rabipur samples showed increased efficiency compared to the other two and was more similar to the ChAd155-RG samples. This animal (Cy14) may have responded to the vaccine particularly well compared to the other two, but with such a small number of individuals it is unclear whether this is an outlier or part of the normal range of Rabipur responses. A larger study would need to be carried out to confirm whether there is a distinct difference in cross-neutralisation between the ChAd155-RG and Rabipur vaccines.

It is clear, however, that a single dose of ChAd155-RG was able to produce an immune response that was comparable to a full 3-dose regimen of Rabipur. This may indicate that an adenovirus-based vaccine is a good contender for a new rabies vaccine that requires fewer boosts in order to provide protection.

Currently, there is an adenovirus-based rabies vaccine (ONRAB) that has been used in Canada in baits to vaccinate animal reservoirs from rabies [247]. This

vaccine has been shown to be safe for use in wildlife and led to protective neutralising antibody titres in 100% of animals 28 days after vaccine administration [62].

There is also a study on potential recombinant human adenoviruses expressing glycoprotein and nucleoprotein genes from a rabies virus isolate as a potential novel rabies vaccine [248]. This study investigated mice vaccinated with single recombinant adenovirus strains or combinations of different strains. The recombinant adenoviruses they produced expressed the signal peptide and ectodomain of the glycoprotein, the whole glycoprotein or the nucleoprotein of a rabies virus isolate. Whilst all vaccinated groups were protected against intramuscular challenge of live rabies virus, the combination of whole glycoprotein with whole nucleoprotein was the only vaccine combination to provide 100% protection against intracranial challenge.

Another study shows that a chimp adenovirus based rabies vaccine is capable of protecting dogs against live rabies infection [249]. In this study the recombinant chimp adenovirus vector expressed the whole rabies glycoprotein of the ERA strain. This vaccine was capable of protecting dogs from lethal rabies infection when given either intramuscularly or orally. This study also used measured the neutralising antibodies following the initial vaccination and after a booster given in week 8, showing a large increase in antibody titre following the boost.

These studies indicate that adenoviruses have been identified as potential vaccine candidates for rabies and can provide protection against rabies infection, though none have yet been licensed for human use.

The additional dose of ChAd155-RG provided as a boost vaccination at week 48 led to an improved neutralisation response compared to the single dose response measured at week 5, with IC_{50} values decreasing by a log. The boosted serum was also capable of neutralising MOKV, which had been resistant to neutralisation with the week 5 serum, though it should be noted that the two

serum samples tested did not have week 5 counterparts so potentially may have shown increased neutralisation in comparison to the other samples.

Future experiments to enhance the data collected here could involve testing all the group 1 serum samples taken after the boost vaccination to obtain more complete data on the improved neutralisation response. MOKV is one of a small number of group 2 lyssaviruses that has been previously shown to have limited levels of cross-neutralisation with group 1 lyssaviruses [69,227], so it would be interesting to know if the boosted serum samples are capable of neutralising other group 2 or 3 lyssaviruses such as LBV or WCBV. It would also be interesting to compare this set of serum samples to the group 2 samples from the NHPs that were vaccinated with ChAd155-RG and then boosted with Rabipur in week 24, to see if the responses are similar.

Additional future work using this vaccine may involve vaccinating animals such as mice with either the novel ChAd155-RG vaccine or Rabipur then infecting them with a live RABV or lyssavirus strain to see how the vaccines compare *in vivo*. Ideally animals vaccinated with Rabipur will also be provided with a boost vaccine after an extended period of time, similar to the ChAd155-RG vaccinated animals in this study, to determine whether the improved neutralisation was specific to the ChAd155-RG vaccine or to the process of providing a boost in general.

In conclusion, in this chapter we looked at a novel chimp adenovirus-based rabies vaccine and used lyssavirus pseudotype assays to compare its neutralisation profile to that of the Rabipur human rabies vaccine. The novel vaccine was able to produce a comparable neutralisation response to Rabipur after a single dose and potentially showed increased cross-neutralisation against other lyssaviruses, particularly after a boost given at week 48.

Chapter 6 – Lyssavirus host species tropism

6.1 Introduction

Viral tropism refers to the specificity of a virus to a particular host species or tissue type. Some viruses are restricted to a single host species such as the smallpox virus, variola virus, which was successfully eradicated in part due to its human-specific tropism and lack of animal reservoirs [250]. Other viruses have many different strains circulating in different animal populations, with one notable example being influenza virus which is well known for having strains circulating in both human and avian populations [251].

Rabies virus has an extremely broad host tropism and is capable of infecting all mammalian species, however it has only established reservoirs in a smaller number of host species such as dogs and other canids, skunks, raccoons and various bat species [252]. Whilst rabies virus has a broad species tropism it has a more restricted tissue tropism and is predominantly a neurotropic virus, traveling through the nervous system to reach the brain [41].

Most lyssaviruses to date have been isolated from various old-world bat species. Little is known about the host tropism breadth of lyssaviruses, with many species having limited numbers of isolations. There are, however, some examples of lyssaviruses causing infections in different animal species. EBLV-1 is predominantly found in *Eptesicus serotinus* bats [253], however spill-over infections have also been reported in sheep in Denmark [14] and a stone marten in Germany [254].

Only two lyssaviruses currently lack a known bat origin. MOKV was originally isolated from shrews in 1968 [10] however the majority of isolations since then have been from domestic cats [255]. As many lyssaviruses have been isolated from bats, it is possible that MOKV may also have a bat reservoir that has yet to be identified. Serum samples taken from fruit bats in Ghana showed similar levels of seroprevalence to MOKV as to LBV, which may be caused by high levels

of cross-reactivity between MOKV and LBV though could also be evidence of prior MOKV exposure [187].

IKOV has a single isolation from an African civet [196], though it is considered unlikely for this to be its reservoir host. Civets are nocturnal, opportunistic foragers and their solitary nature precludes them from acting as an ideal lyssavirus host [90].

As there is currently limited knowledge on the host tropism of lyssaviruses, in this chapter lyssavirus pseudotype infectivity assays were used to infect different animal cell lines in the hope of identifying evidence of host adaptation.

6.2 Results

6.2.1. Lyssavirus infectivity in various animal cell lines

To determine whether the lyssaviruses in the pseudotype panel showed any evidence of adaptation to their animal reservoirs, infectivity assays were performed using cell lines from several animal species known to be lyssavirus reservoirs. The cell lines selected originated from cats (CRFK), *Rousettus aegyptiacus* bats (RoNi/7.1) and *Desmodus rotundus* bats (DesNi). All cell lines were derived from kidney cells to minimise variance from different tissue types.

Lyssavirus pseudotypes were produced as described in Chapter 3. The highly infectious pseudotypes were serially diluted in DMEM to produce standard curves (Fig 6.1). These were then used to calculate appropriate dilutions so that each lyssavirus produced roughly equivalent levels of infectivity in BHK-21 cells (Fig 6.2A) and enabled results to be measured at the same optical gain as the low infectivity pseudotypes (RABV-medoid, RABV-GrD-ev, ABLV-ev and IKOV-ev). Infecting the CRFK cell line led to much higher RLU values than the other cell lines, so much so that the optical gain had to be reduced to 2000 for all lyssavirus pseudotypes in order for luminescence to remain within the linear range of detection. Lower levels of luminescence were observed for the two bat cell lines.

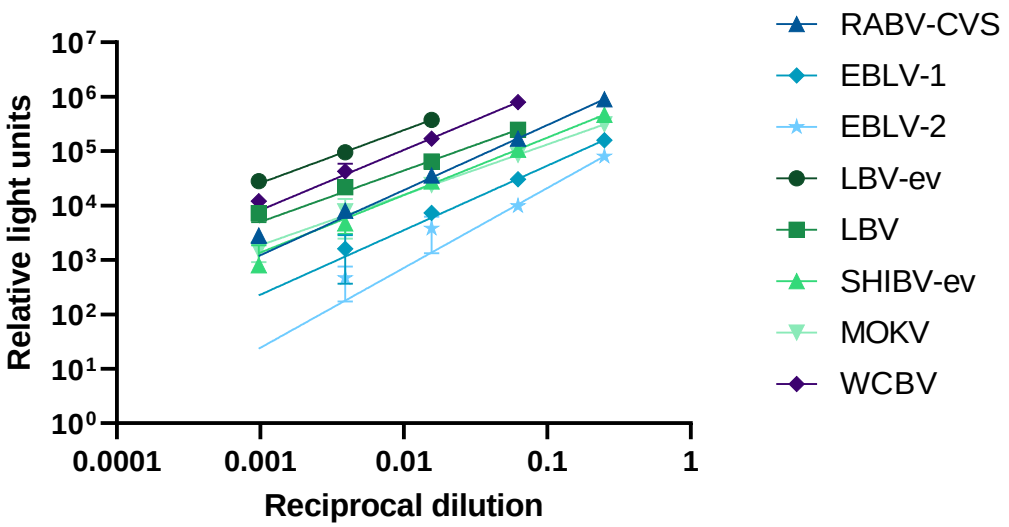


Figure 6.1 High-infectivity lyssavirus pseudotype standard curves
The highly infectious lyssavirus pseudotypes were serially diluted 1 in 4 prior to infecting BHK-21 cells. Results were read at an optical gain of 3,600. ΔEnv negative control values were subtracted to remove the baseline. A non-linear regression line was calculated for the data using Graphpad Prism. Wells were infected in duplicate and the assay was repeated twice with the results presented being from a single, representative experiment. Error bars indicate standard deviation.

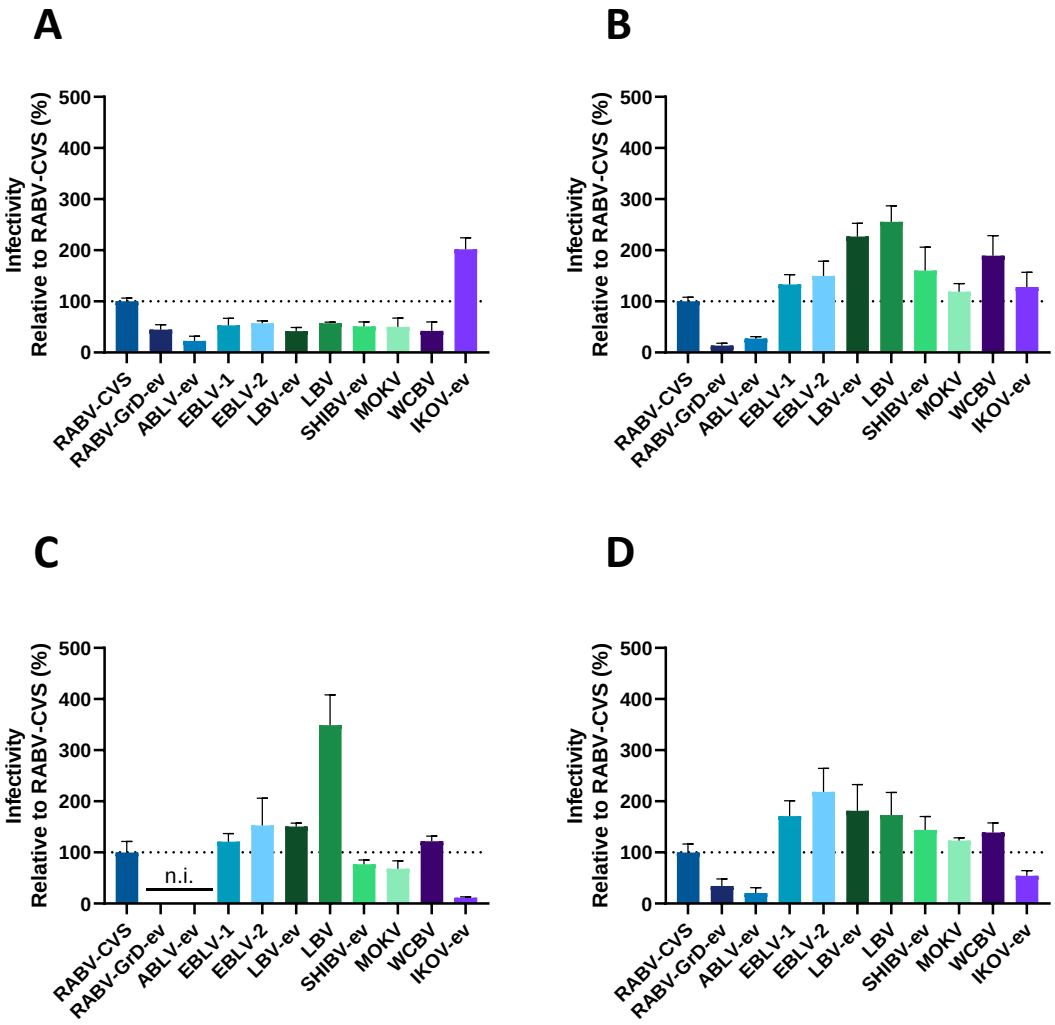


Figure 6.2 Lyssavirus infectivity in animal cell lines
Lyssavirus pseudotypes were created and used to infect different animal cell lines. Results were normalised relative to RABV-CVS in A) BHK-21 cells, B) CRFK cells, C) RoNi/7.1 cells and D) DesNi cells. All pseudotypes were measured at an optical gain of 3,600, with the exception of the CRFK cells where they were all measured at a gain of 2,000 due to increased levels of luminescence. Wells were infected in triplicate and the assay was repeated twice, with the results presented being from a single, representative experiment. The dotted line indicates the level of infectivity achieved by RABV-CVS. Error bars indicated standard deviation. (n.i.: non-infectious)

Results from the infectivity assays were normalised relative to RABV-CVS and results are presented as a percentage of infectivity (Fig 6.2). The RABV-medoid strain was excluded from analysis as it did not show any infectivity in any cell lines other than BHK-21. RABV-GrD-ev and ABLV-ev were not infectious in the RoNi/7.1 cells, with RLU values below the ΔEnv negative control. IKOV-ev showed particularly high infectivity in BHK-21 cells which was markedly reduced in the two bat cell lines relative to RABV-CVS. The remaining lyssaviruses showed increased infectivity in animal cell lines relative to RABV-CVS. The largest increase was seen by LBV in the RoNi/7.1 cell line, which is notable as LBV has been previously isolated from *Rousettus aegyptiacus* [256].

To assess whether there was any correlation in infectivity between the cell lines, Pearson's correlation was performed on pairs of cell lines. There was a positive correlation seen in infectivity between the three animal cell lines with the exception of RoNi/7.1 and DesNi (Fig 6.3). This is likely due to the presence of LBV as an outlier, showing a large increase in infectivity in the RoNi/7.1 cells but not in the DesNi cells. The other lyssaviruses showed a noticeable positive correlation in these two cell lines. When LBV was removed from the analysis the r^2 increased to 0.78 with a p value of 0.0034.

When originally producing the lyssavirus pseudotype panel, a rabies virus identified within a vampire bat (RABV-GtB-ev) failed to produce viable pseudotypes despite attempts at optimisation and mutagenesis (Chapter 3). To determine whether this virus was capable of infecting cells from its original host, pseudotypes of this virus and the SDM mutated virus (RABV-GtB-ev-A159C) were produced and used to infect the CRFK, RoNi/7.1 and DesNi cell lines. However, regardless of which cell line was used, this pseudotype did not produce any infectivity higher than the ΔEnv positive control (Fig 6.4).

6.2.2. Lyssavirus receptor expression in cell lines

When selecting cell lines for use in the lyssavirus tropism experiments an important consideration made was the original tissue source of the cells. All cell lines chosen were originally from kidney cells, to reduce any variability in

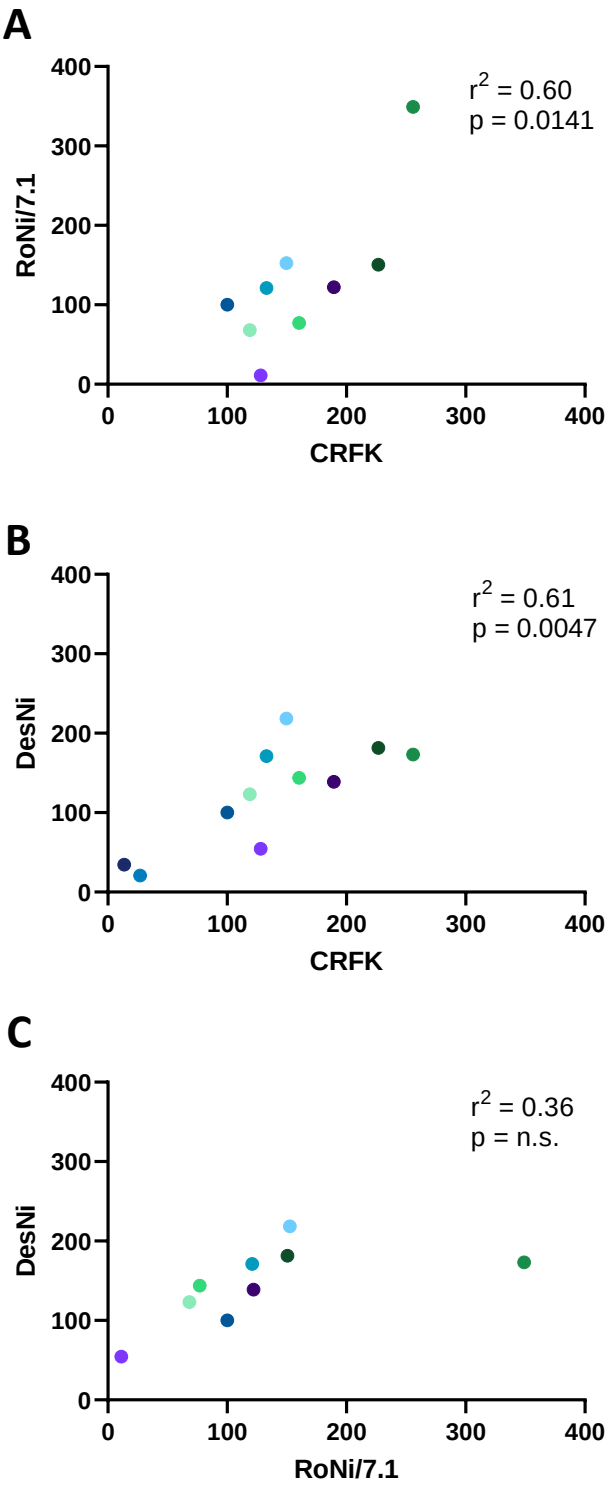


Figure 6.3 Correlation of infectivity between cell lines
Mean values of the normalised infectivity data for the three animal cell lines were plotted and correlations calculated using Pearson's correlation test. (n.s.: not significant)

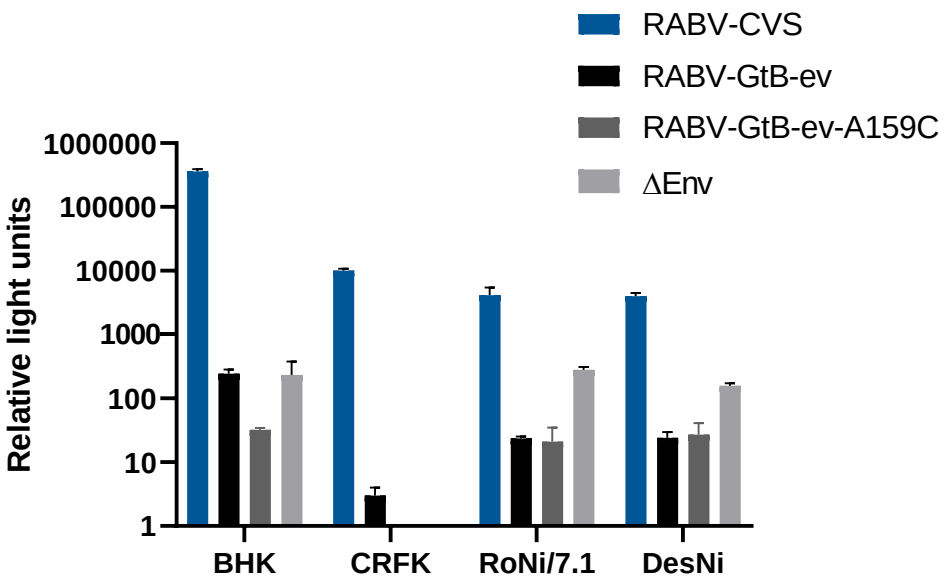


Figure 6.4 Infectivity of RABV-GtB-ev in animal cell lines
Pseudotypes were produced for the RABV-GtB-ev and mutated A159C strains and used to infect different animal cell lines. RABV-CVS was included as a positive control, with ΔEnv pseudotypes as a negative control. Wells were infected in triplicate and the assay was repeated twice with the results presented being from a single, representative experiment. Error bars indicate standard deviation.

infectivity that may be caused by differing receptor expression profiles. However, the original organs were not formed from one homogenous cell type and the DesNi cells in particular are a heterogeneous mixture of different cell types [257] rather than a clonal cell line such as the CRFKs. As such, each cell line may still display different receptor expression profiles.

RT-qPCR was performed in order to determine approximate levels of mRNA expression of the three main potential lyssavirus receptors. Primers to amplify *GAPDH* and *HPRT1* housekeeping genes were identified from the literature [200,201]. Multiple sequence alignments were created for the *CHRNA1* (nAChR α1 subunit), *NCAM1* (NCAM/CD56) and *NGFR* ($p75^{NTR}$) genes with human, hamster, cat, fruit bad and vampire bat sequences (Fig 2.3). Primers were designed from conserved regions to reduce mismatches and avoiding gaps in the alignment to ensure all products would be identical sizes (Appendix I). Primers were situated on separate exons to determine whether products originated from mRNA or genomic DNA contamination.

RNA was extracted from approximately 6,000 cells from each cell line and 3 μg RNA was converted to cDNA using random hexamers. cDNA was used in qPCR using SYBR green to detect the rate of product formation.

PCR products were assessed by running the final products on a gel to confirm that only a single product was produced in each reaction. Whilst *HPRT1* was cleanly amplified from all cell lines tested, *GAPDH* gave multiple bands for the RoNi/7.1 cells (Fig 6.5). *CHRNA1* and *NCAM1* were successfully amplified from both BHK-21 cells and CRFK cells, but not from either of the bat cell lines. NGFR was amplified from the CRFK cells but multiple products were seen for BHK-21 cells and RoNi/7.1 cells, with no products observed for the DesNi cells. As such, only *CHRNA1* and *NCAM1* expression could be compared between BHK-21 cells and CRFK cells.

As there were two housekeeping genes available, expression of the two receptor genes was assessed relative to both *GAPDH* and *HPRT1* using the $2^{-\Delta\Delta Cq}$ method

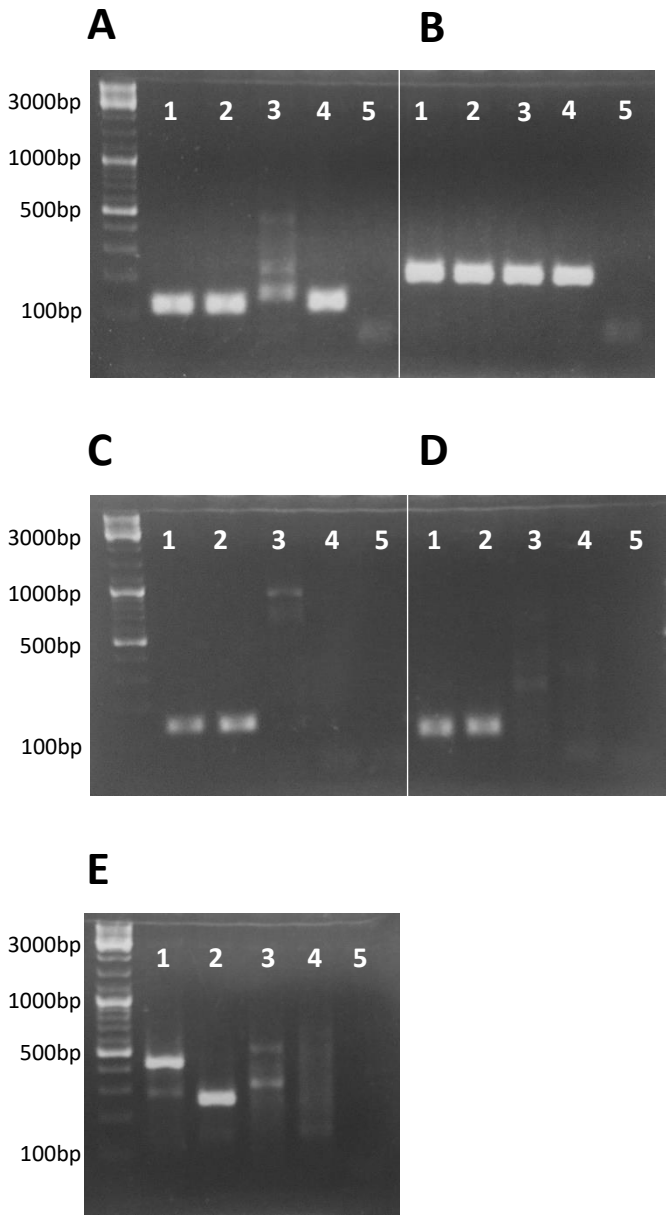


Figure 6.5 PCR products from housekeeping genes and putative lyssavirus receptors

Detection of PCR products for the genes A) *GAPDH* (product size: 117 bp), B) *HPRT1* (product size: 195 bp), C) *CHRNA1* (product size: 139 bp), D) *NCAM1* (product size: 136 bp), E) *NGFR* (product size: 254 bp). Cell lines are indicated by number with 1) BHK-21, 2) CRFK, 3) RoNi/7.1, 4) DesNi, 5) no template control. The PCR was performed a single time.

(Fig 6.6). Whilst both normalisations indicated lower levels of *NCAM1* expression in CRFK cells relative to BHK-21 cells, the expression of *CHRNA1* varied dependent on housekeeping gene used. Variability of the housekeeping genes was displayed using box and whisker plots (Fig 6.7) [258] and indicated that *GAPDH* showed increased variation in C_q value across cell lines than *HPRT1*.

6.3 Discussion

A range of animal cell lines were selected for the tropism infectivity assays based on their relevance to lyssavirus infection. The cell lines chosen originated from cats, *Rousettus aegyptiacus* old-world frugivorous bats and *Desmodus rotundus* new-world haemotophagous bats. All three of these species are known to be capable of harbouring infections from different lyssaviruses, so it was hypothesised that these lyssaviruses may show increased infectivity in cell lines originating from their hosts.

Tropism infectivity experiments showed that the various lyssaviruses each had different infectivity profiles in the different animal cell lines. The most striking example is that LBV was approximately 3 times more infectious in the RoNi.7.1 cells than RABV-CVS. The LBV-ev and EBLV-2 lyssaviruses also showed a slight increase in infectivity relative to RABV-CVS. LBV has been previously isolated from *Rousettus aegyptiacus* bats [256], though it has been more commonly associated with *Eidolon helvum* frugivorous bats [259].

As the LBV strain had been isolated following intracerebral inoculation into mice and passaged multiple times through mouse brains prior to sequencing [192,260], it was originally hypothesised that this strain would show less infectivity in cells from a bat reservoir than the LBV-ev strain that was sequenced directly from an infected bat brain [199]. Whilst both strains showed increased infectivity in the RoNi/7.1 cells relative to RABV-CVS, this did not prove to be the case, with LBV showing a much greater increase in infectivity than LBV-ev.

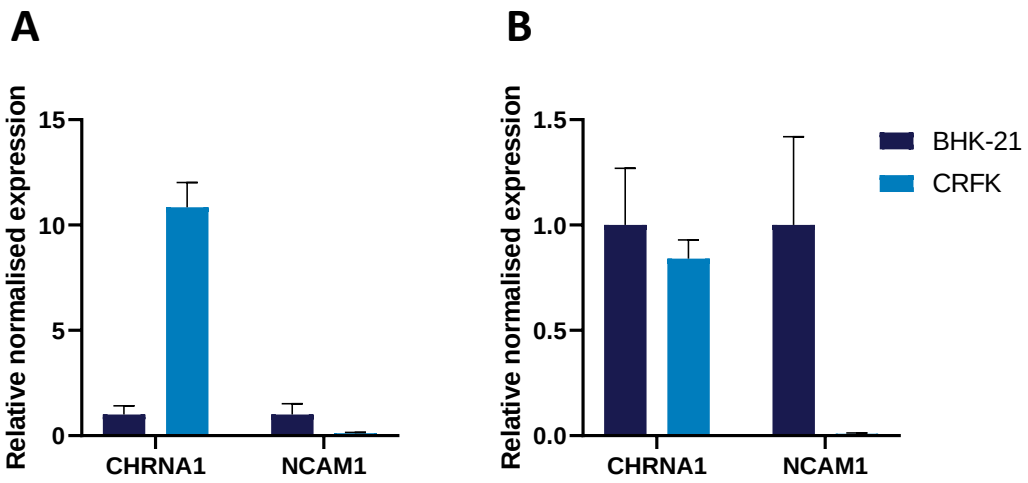


Figure 6.6 Relative gene expression of *CHRNA1* and *NCAM* in CRFK and BHK cells
Gene expression of *CHRNA1* and *NCAM1* was assessed in BHK-21 and CRFK cells using SYBR green RT-qPCR. Genes expression was normalised relative to A) *GAPDH* and B) *HPRT1* using the $2^{-\Delta\Delta Cq}$ method. Samples were tested in duplicate and the qCPR was performed a single time. Error bars indicate standard deviation.

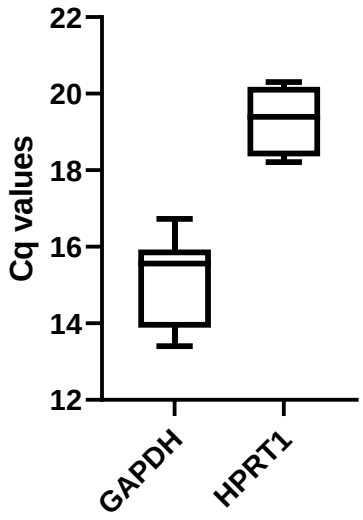


Figure 6.7 Variability of C_q values from housekeeping genes across all cell lines

Box and whisker plots indicating the range of C_q values for the housekeeping genes *GAPDH* and *HPRT1* with the central line indicating the median and the whiskers showing the range of values. *GAPDH* C_q values were only assessed for BHK-21, CRFK and DesNi cells, whilst HPRT1 C_q values also included results for the RoNi/7.1 cells.

One possible explanation for this is that both LBV strains in this study came from different LBV lineages. Lyssaviruses were originally assigned to serotypes based on serological cross-reactivity [92], however as molecular biology techniques have advanced lyssaviruses have been separated into genotypes based on genetic variation [93]. LBV was one of the first non-RABV lyssaviruses to be identified, along with MOKV [10] and has been isolated multiple times since it was first discovered in 1956. As such, multiple isolates have been classified as belonging to the LBV strain based on their serotype rather than their genotype. Phylogenetic analysis of LBV isolates has suggested that LBV can be separated into at least three distinct lineages, with lineage A showing sufficient diversity to be reasonably considered a separate lyssavirus genotype [192]. Whilst the lab-adapted LBV strain used in this chapter belongs to lineage B, the LBV-ev strain clusters with lineage A LBV viruses (Fig 6.8). Lineage A LBV strains have been suggested to have distinct characteristics from other LBV strains such as increased pathogenicity in laboratory animals [261] which may explain why LBV-ev infectivity in RoNi/7.1 cells did not increase to the same extent as LBV.

MOKV is a lyssavirus that has been isolated multiple times from cats [255] and so it was predicted to show an increased infectivity in the CRFK cells. As seen in figure 6.2, MOKV did indeed show a slight increase in infectivity in the CRFK cell line relative to RABV-CVS, though it also showed a similar level of increase in the DesNi cells as well. This may support the theory that MOKV has an unknown bat origin, though as the DesNi cells are new-world bat cells this does not help to narrow down potential reservoir species. The infectivity profile of MOKV was fairly similar to that of SHIBV-ev, however, which was isolated from an insectivorous bat, *Hipposideros commersoni* [194], so there is the potential for MOKV to have originated in insectivorous bats. Without an isolation from an insectivorous bat or infectivity data from an insectivorous bat cell line, this remains purely speculative.

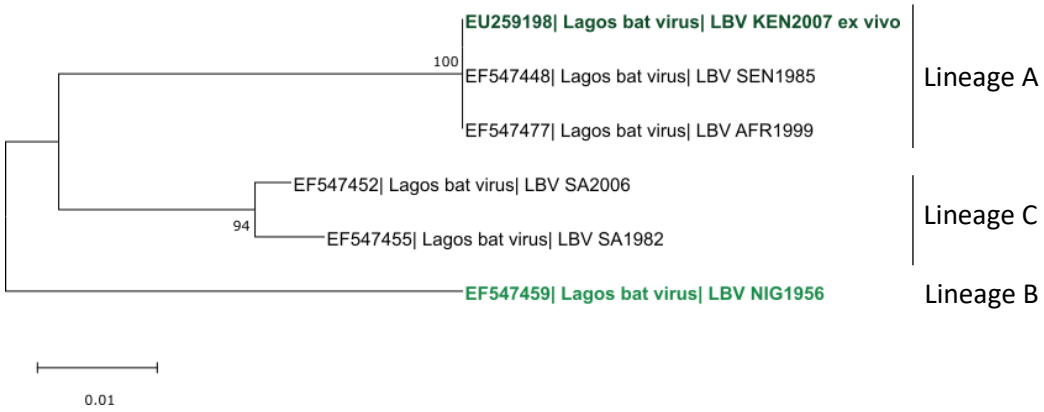


Figure 6.8 Phylogenetic tree of Lagos bat virus nucleoprotein amino acid sequences

The maximum likelihood tree was generated using a JTT+G+I model in MEGAX. Tree topology was assessed using bootstrap analysis of 500 replicates. Bootstrap percentages are shown at relevant nodes. The scale bar indicates the number of amino acid substitutions per site. LBV strains used in the lyssavirus pseudotype panel have been coloured accordingly.

IKOV-ev is another lyssavirus with an unknown reservoir host and had a distinct infectivity profile from the other lyssaviruses. IKOV-ev showed slightly reduced infectivity in CRFK cells relative to RABV-CVS and a markedly reduced infectivity in both the RoNi/7.1 and DesNi bat cells. The reduction in infectivity is particularly noticeable as whilst raw RLU values for the two bat cells were lower for all lyssaviruses across the board, IKOV-ev originally had the highest RLU values in the BHK-21 cells as it was not diluted with DMEM prior to exposure to cells.

The single isolation of IKOV came from an African civet, which is not considered likely to be the reservoir host due to its solitary nature [90]. As such, it has been typically assumed to originate from bats similar to the other group 2 and 3 lyssaviruses, but the data from this chapter may indicated that this is not the case. Civets are omnivores that eat small vertebrates, so there is the possibility that IKOV may have a rodent host. Previous isolations of MOKV from shrews [10] indicates that rodents are capable of being naturally infected by lyssaviruses, but their status as a reservoir host is questionable.

RABV-GtB-ev is a rabies virus strain that was sequenced directly from brain tissue of an infected vampire bat in Guatemala [213]. Attempts to pseudotype this virus strain in Chapter 3 failed despite attempts at optimisation and mutagenesis. It was hypothesised that this strain may only be capable of infecting its original bat host, however both this strain and the mutated strain RABV-GtB-ev-A159C failed to infect any of the cell lines. The reasons for the pseudotype failure are unknown, however could be linked to differences in glycoprotein density on the pseudotyped particles compared to on wild type viruses. The RABV matrix protein interacts with the glycoprotein when assembling virions and is responsible for RABV's characteristic bullet shaped morphology [109]. As RABV-GtB-ev was isolated from an infected bat, it is clearly an infectious RABV strain, so potentially could require the presence of the RABV M protein to be incorporated efficiently into the budding virus particles.

Whilst the infectivity assays provide some tantalising hints regarding the tropism of the lyssaviruses present in the pseudotype panel, there are limitations that must be considered. Firstly, the raw RLU values cannot be compared directly between cells as there are various factors that may influence luminescence output unrelated to pseudotype infectivity.

As the different cell lines displayed different growth characteristics, they had to be seeded into the 96-well plates at different densities to remain healthy. Whilst the BHK-21 cells were seeded at a density of 20,000 per well, the other cell lines were seeded at 15,000 per well. This automatically leads to variability as it is highly likely that different numbers of cells would be transduced by the pseudotyped viruses between the different cell lines.

Another issue is that different cell lines can be transduced with different levels of efficiency [262], which may be caused by factors within the cell rather than being related to virus tropism [263]. Cells from specific animal species may contain restriction factors against retroviral or lentiviral vectors [212,264], however this would presumably affect all pseudotyped viruses equally.

Due to the difficulties in comparing infectivity across cell lines, results were normalised in order to display infectivity as a relative percentage. RABV-CVS was chosen as the pseudotype virus to compare the other lyssaviruses to firstly because it was a RABV strain and should therefore display broad tropism, but also because it is a lab-adapted virus strain with an extensive passage history so would presumably lack specific host tropism.

The receptor expression profile was also briefly explored in this chapter, however unfortunately the primers designed were not able to amplify the three receptors in all four cell lines. This limited the comparison to the BHK-21 and CRFK cell lines and to the expression of *CHRNA1* and *NCAM1*.

The results of the RT-qPCR show the importance of validating housekeeping genes for the cell lines tested, as the relative expression of the *CHRNA1* gene

varied considerably depending on whether results were normalised to *GAPDH* or *HPRT1*. There are various methods available for assessing the variation of housekeeping gene expression, however it is no easy task to identify appropriate housekeeping genes, so a handful of housekeeping genes tend to be used [265]. Identifying and validating reliable housekeeping genes was beyond the scope of the work in this chapter, so two common housekeeping genes were selected based on the identification of primers from the literature. The *GAPDH* primers had been originally designed to target the hamster version of the *GAPDH* gene and so were ideal for use in the BHK-21 cells [200]. The *HPRT1* primers, on the other hand, had been explicitly designed as universal primers that should theoretically work in a variety of animal species [201].

Both sets of housekeeping primers worked well across the different cell lines, with the *GAPDH* primers falling behind due to their failure to cleanly amplify *GAPDH* in the RoNi/7.1 cells. When looking at the raw C_q values, the *GAPDH* primers also showed a greater range of values across successfully amplified cell lines in comparison to *HPRT1*. It should be noted, however, that this may not be due to differences in expression levels but could be related to differences in PCR efficiency or starting cDNA concentrations.

As such, future work is required in order to draw any solid conclusions regarding receptor expression across the animal cell lines, though it seems likely that the NCAM1 receptor is expressed to a much lower extent in the CRFK cells than the BHK-21 cells. Whilst it would be ideal to use the same set of primers for all cell lines, individual primer sets for each animal species may be required to amplify all of the receptors as the receptor genes were quite variable, particularly *NCAM1*. Standard curves should be produced for each primer set to estimate the efficiency of the reaction and to confirm that the typical $2^{-\Delta\Delta Cq}$ normalisation calculation is appropriate. An additional housekeeping gene may need to be investigated to compare with *GAPDH* and *HPRT1*.

Another option would be to investigate receptor expression directly through the use of immunofluorescence, though suitable antibodies would need to be identified against the target receptors.

To further investigate the impact each receptor has on lyssavirus infectivity, receptor knock out cell lines could be generated. There are several methods available to develop gene knockout cell lines, such as homologous recombination and site-specific nucleases.

Homologous recombination involves creating a construct that contains the gene with the desired mutation and delivering this construct to the cell where it can be recombined with the cell's DNA. A selection marker is also included in the construct to allow for proliferation of cells that have successfully integrated the mutated gene [266]. Homologous recombination is a specific tool that can introduce both large and small changes into a genome, however the main drawback is the low rate of gene targeting making it inappropriate for use in mammalian cells [266].

Site-specific nucleases work by targeting a DNA sequence inside the cell and introducing a double-stranded break. When the cell repairs the break there may be insertion or deletion of bases, leading to frameshift mutations. There are several different methods available involving site-specific nucleases such as zinc-finger nucleases and clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 [267].

Rather than generating a knockout cell line, it is also possible to use siRNA to transiently reduce protein expression in cells prior to performing infectivity assays [268]. This would have the advantage of being a simple procedure, requiring a transfection rather than the more involved process of generating a knockout cell line. There are disadvantages to this approach though, such as off-target effects and inefficient knockdown preventing complete loss of gene function [269].

Potential receptors could also be expressed in cell lines through transfection of plasmids containing the required gene plus a selection marker or through lentiviral transduction. Lentiviral transduction leads to more efficient generation of stably-transfected cells than plasmid transfection, however there may be issues with virus-mediated immunogenicity and viral vectors have limitations on the size of genes they can effectively transfer, with viral titres reducing as the insert size increases [270].
Once suitable cell lines are generated, they could be used in lyssavirus infectivity assays to determine the effects of each of the proposed receptors on the different lyssavirus species, potentially helping to clarify the impact each receptor has on virus entry.

In conclusion, in this chapter lyssavirus infectivity profiles were investigated using multiple animal cell lines and potentially identified evidence of host adaptation, though the results could not be correlated to receptor expression and further work is required in this area.

Chapter 7 - Discussion

Rabies encephalitis is a neglected tropical disease with a mortality rate approaching 100% once symptoms have developed [42], leading to an estimated 60,000 deaths per year predominantly in poor, rural areas where there may be limited accessibility to treatment [28,29]. Whilst >99% of all human rabies cases involve the transmission of rabies virus from dogs [3], other lyssaviruses are also capable of causing rabies encephalitis [74].

Vaccination and administration of anti-rabies immunoglobulins are currently the only treatments available for protecting against rabies and treatment is only effective when provided prior to the development of symptoms. The WHO recommends a schedule of up to five vaccine doses administered across a 28 day period for effective post-exposure prophylaxis [234]. This lengthy treatment regimen may help to contribute to prophylaxis failure, with full treatment schedules not being provided due to lack of supply or treatment costs being prohibitive, thus increasing the risk of developing fatal rabies [236]. As such, more immunogenic rabies vaccines are desirable to reduce the number of doses required.

Current vaccines provide only limited levels of protection against lyssavirus infection [73,180,227,228] and whilst to date only a small number of human lyssavirus-induced rabies cases have been reported, the causative agent of a rabies infection is often not identified as some clinical tests are unable to distinguish between lyssavirus genotypes [271]. Lyssaviruses can be found worldwide, however with limited isolations for each genotype, information on their host reservoirs is lacking.

This study aimed to use a lyssavirus pseudotype panel to investigate the neutralising antibody response generated by current and novel rabies vaccines against various lyssaviruses and to look for evidence of host species tropism through comparing infectivity profiles in different animal species.

The modular pseudotyped virus system offers the flexibility to easily create pseudotypes from a wide variety of different viruses through swapping out plasmids bearing different envelope glycoproteins. In Chapter 3, a panel of lyssavirus pseudotypes was generated using both *ex vivo* and lab-adapted virus strains. During the course of development, it was identified that the pI.18 plasmid vector was more suitable than the pcDNA3.1 vector for the expression of lyssavirus glycoproteins, leading to greatly increased pseudotype infectivity for the majority of strains tested.

Different promotors present in different plasmid vectors can have an effect on envelope glycoprotein expression [272] and choice of plasmid has been previously shown to affect pseudotype infectivity [208]. However, the two expression plasmids used in this study, pcDNA3.1 and pI.18 both used the CMV IE promotor therefore there is no obvious reason for the difference in pseudotype infectivity displayed by these two vectors. One possibility is that the intron A sequence present in the pI.18 vector may be enhancing protein expression [220] however this would need to be inserted into the pcDNA3.1 vector for confirmation.

Optimisation of relative plasmid amounts has previously been shown to be a method to increase infectivity for viruses that are tricky to pseudotype [182], but this method had little effect on pseudotype infectivity for the lyssaviruses tested in this study.

The development of the lyssavirus pseudotype panel in Chapter 3 enables the study of a broad range of lyssavirus species. The pseudotype panel was based on an MLV backbone as opposed to the HIV-1 backbone that has been used in previous lyssavirus pseudotype studies. This allows for the use of pseudotypes in cells which are non-permissive for lentiviral vectors, such as bat cells and N2A cells [180,184]. The data produced in this chapter also provides further information on how to optimise the pseudotyping process, whether through adjusting the amounts of plasmids transfected or switching plasmid vectors.

This could help to inform future research and aid in the development of additional pseudotype panels for other viruses.

Further research using the lyssavirus pseudotype panel could include work to improve understanding on how the pseudotypes work, such as through looking in greater detail at the expression plasmids and how infectivity is affected by the presence of the intron A sequence. Protein expression could also be examined through developing effective Western blots which may indicate if the differences in infectivity between the two plasmids was due to increased protein expression.

The lyssavirus pseudotype panel was used in Chapters 4 and 5 to investigate lyssavirus neutralisation by vaccinated serum. Neutralisation assays using the full lyssavirus pseudotype panel treated with vaccinated human serum confirmed previous research that neutralisation is common across all lyssaviruses within phylogroup 1, but does not extend to the more genetically distant viruses of phylogroups 2 and 3 [73,180,227,228].

Glycosylation mutants of the phylogroup 1 lyssaviruses were also generated with the aim of determining whether the presence of a glycosylation site at position 204 would have an effect on lyssavirus infectivity and neutralisation. It was found that the addition of this glycosylation site led to an increase infectivity for most of the strains tested, but didn't have an impact on others. It appeared that the presence of this glycosylation site had little effect on the neutralisation curves of the lyssavirus strains tested, though ideally this work would be repeated due to the data quality for this experiment being low.

The glycosylation mutants generated in this study still have a lot of work that could be performed to elucidate the effects of this glycosylation site on both infectivity and neutralisation. The mutated strains could be used to infect different cell lines to see whether the presence of this glycosylation site had an effect on virus tropism as well as infectivity. Western blots could also be used to

determine whether the additional glycosylation site was actually being glycosylated.

Serum taken from non-human primates (NHPs) vaccinated with a novel chimp adenovirus-based rabies vaccine (ChAd155-RG) was compared to serum from NHPs vaccinated with a current human rabies vaccine (Rabipur) using a pseudotyped virus based neutralisation assay to determine whether it invoked a comparable neutralising antibody response. Results indicated that a single dose of the novel vaccine was capable of eliciting a comparable response to the Rabipur vaccine after a full three dose vaccination schedule.

The ChAd155-RG vaccine also may have been slightly more effective against non-RABV lyssaviruses than the Rabipur vaccine, though a larger study would be required to confirm this. A boost vaccination provided after 48 weeks was capable of greatly increasing the neutralising antibody response and allowing for cross-neutralisation of the group 2 lyssavirus MOKV. Adenoviruses are currently being explored for their suitability as rabies vaccine vectors [62,248,249] and the results from Chapter 5 provide support for their high immunogenicity and may indicate that the ChAd155-RG vaccine is a good candidate for further development.

Additional neutralisation experiments could be performed using the NHP serum samples, in particular to look in more depth at the effect of the boost vaccination in week 48 by investigating the neutralisation against more diverse group 2 and 3 lyssaviruses and to compare with the ChAd155-RG vaccinated serum that received a boost with Rabipur in week 24.

Pseudotyped viruses can also be used to investigate both tissue tropism [273] and host species tropism [184] by looking at how pseudotype infectivity varies across different cell lines. In Chapter 6 the lyssavirus pseudotype panel was assessed for any evidence of host adaptation by infecting animal cell lines taken from cats, frugivorous bats and haematophagous bats.

The LBV strain showed an increase in infectivity relative to RABV-CVS in the frugivorous bat cell line, which is notable as LBV has been previously isolated from cells of this bat species [256]. Increases were also seen in the cat and haematophagous bat cell lines, which may indicate that this genotype has a broad host tropism. The LBV-ev strain however showed a much more modest increase in the frugivorous bat cell line, which may be due to its different lineage. Another potential example of host adaptation is that the MOKV strain showed an increase in infectivity in the cat cell line relative to RABV-CVS, with a similar level of increase also present in the haematophagous bat cells. The IKOV-ev strain, however, had an infectivity profile unlike any other lyssavirus, with a slight increase in relative infectivity in the cat cells but a prominent reduction in relative infectivity in the bat cells. As the reservoir for IKOV has yet to be identified this is an interesting observation and more cell lines from other species need to be tested to potentially indicate the original reservoir host.

These results help to provide additional information on a variety of diverse lyssaviruses in a range of animal species. Knowledge of the infection capabilities of non-RABV lyssaviruses is sadly limited and this data helps to expand upon that. The dramatic increase in infectivity shown by LBV in the frugivorous bat cells demonstrates that despite the broad host tropism shown by RABV and presumably all other lyssaviruses, this assay can distinguish differences in affinity for different host species.

Future experiments to investigate lyssavirus tropism could involve additional infectivity assays on cell lines of interest, such as a dog cell line, cells from livestock species such as cows or sheep, and human cells. This could help to identify lyssaviruses that have an increased potential to cross into a new species reservoir.

Receptor expression in the cell lines could be investigated through optimising the RT-qPCR by designing additional primers and producing standard curves. Immunofluorescence or western blots could also be performed to confirm receptor expression. Another interesting direction to go in could be to produce

receptor knockouts or stable-expression for a few different cell lines to see how the infectivity of the lyssavirus pseudotypes is affected. These experiments would help to show the impact of different receptors on each of the lyssavirus strains and provide further clarity on the role each receptor plays in viral entry.

Overall, the results obtained throughout this study confirmed and built upon those shown by other groups and the repeatability of individual experiments was typically good. Whilst the raw RLU values of the pseudotyped viruses may vary from batch to batch, the differences between the lyssavirus strains were generally constant, with the LBV-ev and WCBV strains showing the highest levels of infectivity and the RABV-medoid, RABV-GrD-ev and ABLV-ev strains showing the least.

Pseudotype batch variability did however cause issues throughout the project, as whilst it didn't have a major impact on the highly infectious pseudotypes, the less infectious strains were very sensitive to culture conditions and RLU values could easily reduce to unusable levels. Problems would typically arise due to quality of the transfection reagent. As this was made up in-house, it could be prone to batch variability itself and transfection efficiency would slowly decrease over time when stored for long periods. Cell cultures reaching high passage numbers could also lead to a reduction in pseudotype quality. A lot of time was spent throughout the project troubleshooting the less infectious pseudotype strains when they lost infectivity, in an attempt to discover where the issue was coming from. Unfortunately, the less infectious strains could not be frozen for long term storage as this also led to a reduction in infectivity, so they always needed to be made up fresh.

These problems could be reduced by either only working with the highly infectious pseudotype strains or through diligence in maintaining cell health and confirming the efficiency of every batch of transfection reagent.

Another issue regarding the pseudotype infectivity was that there was such a large difference in infectivity between the highly infectious and less infectious

strains that it could be difficult to compare the two sets. The neutralisation assays all involved normalising against an untreated control, so there was no requirement for the lyssaviruses to all be measured at the same optical gain on the plate reader provided there was enough dynamic range in infectivity between the lyssavirus and the ΔEnv negative control. However, for the tropism experiments there needed to be some way to compare strains which had vastly different levels of infectivity. The highly infectious strains were therefore diluted in order to allow results to be measured at the same settings as the less infectious strains.

One possibility to standardise the pseudotype luminescence output would be to measure the $TCID_{50}$ and dilute each strain in order to infect cells with an equivalent dose. This can be done by performing serial dilutions on the pseudotype strains and then performing a calculation such as the Reed-Muench method or Spearman-Kärber method [274]. Such calculations are traditionally performed to determine the titre of live viruses [275], though they have also been used in the quantification of pseudotyped viruses [180].

The neutralisation assays performed in this study were very repeatable and neutralisation curves were consistent across different batches of pseudotypes. There could be a fair amount of variability in replicates when the pseudotypes were treated with serum and this was particularly noticeable in the pre-vaccination serum experiments, leading to some wide error bars.

One minor issue that cropped up when performing the neutralisation assays was that pseudotype infectivity often wouldn't return to 100% infectivity even at very low concentrations of serum, plateauing at around 80-90% infectivity. This was particularly noticeable in the NHP experiments and is likely a technical issue caused by positioning the untreated controls along the top row of the 96-well plate, possibly causing them to be subject to the edge effect, artificially inflating the infectivity values. As full neutralisation curves were still clearly visible for all pseudotypes tested, this was considered to be a relatively minor

issue, but it could potentially be alleviated by adding the untreated controls to a separate plate.

In conclusion, pseudotyped viruses are a useful tool for exploring viral entry and their modular nature allows for the development of large numbers of different viruses through the substitution of the glycoprotein plasmid. Over the course of this project a lyssavirus pseudotype panel has been developed and used to investigate differences in neutralisation between the lyssaviruses, differences in neutralisation caused by different vaccines, and infectivity in different animal cells. These experiments show the flexibility of the pseudotype platform and its utility in investigating viral entry and neutralisation, providing promising data regarding a novel lyssavirus vaccine and producing insights into the species tropism of diverse lyssavirus strains.

Appendix I – PCR primers

Name	Sequence	Annealing temp (°C)
TOPO cloning primers		
pcDNA_RabF	CACCATGGTGCCTCAAGC	60
pcDNA_RabR	TCACAGCCTGGTCTCTCC	
RABV_Gr_DogF	CACCATGGTTCCTCAAGCTC	58
RABV_Gr_DogR	TCACAGTCTGGTCTCACCC	
RABV_Gt_BatF	CACCATGATCCTTCAGGTCCTTC	58
RABV_Gt_BatR	TCACAGCCTGGCATCGC	
LBV_Ken_BatF	CACCATGAGTCAATTGTTCTCAAC	58
LBV_Ken_BatR	TTAGACACTTGATGTCCCTTTATATGAC	
CVS_11_Fwd	CACCATGGTTCCTCAGGTTCTTTTGTTTG	58
CVS_11_Rev	TCACAGTCTGATCTCACCTCCAC	
InFusion cloning primers		
InfRab_pI_F	CAGTGTGGTGGAATTCCACCATGGTGCCTCAAGCCCT	58
InfRab_pI_R	GATATCTGCAGAATTCTCACAGCCTGGTCTCTCC	
InfGr_Dog_pI_F	CAGTGTGGTGGAATTCCACCATGGTTCCTCAAGCTCTTTTG	56
InfGr_Dog_pI_R	GATATCTGCAGAATTCTCACAGTCTGGTCTCACC	
InfGt_Bat_pI_F	CAGTGTGGTGGAATTCCACCATGATCCTTCAGGTCCTTCAG	56
InfGt_Bat_pI_R	GATATCTGCAGAATTCTCACAGCCTGGCATCG	
ABLV_InfA_Bat F	CAGTGTGGTGGAATTCCACCATGCTCCTTCAAATTGTCCTCC T	60
ABLV_InfA_Bat R	GATATCTGCAGAATTCTCACGATCTGAAATGACCTCC	
InfK_Bat_pI_F	CAGTGTGGTGGAATTCCACCATGAGTCAATTGTTCTCAACC TTC	57
InfK_Bat_pI_R	GATATCTGCAGAATTCTTAGACACTTGATGTCCCTTTATATG	
InfEBLV_1_Fwd	CAGTGTGGTGGAATTCCACCATGTTACTCTCTACCGCCATAT TC	58
InfEBLV_1_Rev	GATATCTGCAGAATTCTTATGACTCACCAGTGGCC	
InfEBLV_2_Fwd	CAGTGTGGTGGAATTCCACCATGCCATTCCAAGCTGTTTTG	60
InfEBLV_2_Rev	GATATCTGCAGAATTCTTAAGACTGACCCCCCGTC	
InfLBV_Fwd	CAGTGTGGTGGAATTCCACCATGAGTCAACTAAATTTGATA C	50
InfLBV_Rev	GATATCTGCAGAATTCTCAGACATTAGAGGTACC	
InfMOKV_Fwd	CAGTGTGGTGGAATTCCACCATGAACCTACCCTGTCTGAC	58
InfMOKV_Rev	GATATCTGCAGAATTCTCATGTACCAGGGAGCCC	
InfSHIBV_Fwd	CAGTGTGGTGGAATTCCACCATGTCCAACCTGTGCACTATC	58
InfSHIBV_Rev	GATATCTGCAGAATTCTTACACTGAAGATGTGCCTTTGT	

Name	Sequence	Annealing temp (°C)
InfWCBV_Fwd	CAGTGTGGTGGAATTCCACCATGGCTTCCTACTTTGCGTTG	59
InfWCBV_Rev	GATATCTGCAGAATTCTTATTGGGCAGTTTGTCCCTTG	
InfIKOV_Fwd	CAGTGTGGTGGAATTCCACCATGGCACAGCTGGTGACTC	60
InfIKOV_Rev	GATATCTGCAGAATTCTTAAAAGGCACTTGATTTGTAGGAC TC	
InfVSV_Fwd	CAGTGTGGTGGAATTCCACCATGAAGTGCCTTTTGTACTTA GC	57
InfVSV_Rev	GATATCTGCAGAATTCTTACTTTCCAAGTCGGTTCATC	
	Plasmid sequencing primers	
T7Fwd	TAATACGACTCACTATAGGG	55
bGHRev	TAGAAGGCACAGTCGAGG	
pI.18Fwd	TCCATGGGTCTTTTCTGCAG	60
pI.18Rev	CAGGCGTGACACGTTTATTG	
	SDM primers	
pI.18_SDM_F	TTCTGCAGATATCCTCGAGGTATTCTAGATCCCACGTCA	67
pI.18_SDM_R	TTCCACCACACTGGCGGTACCCGGATCCGAT	
pI.18_SDM_F3	AATTCTGCAGATATCCTCGAGGTATTCTAGATCCCACGTCA	67
pI.18_SDM_R3	CCACCACACTGGCGGTACCCGGATCCGAT	
Gt_Bat_SDM_F	CTGGCGGAAGTTGTTTGGGAATAACG	61
Gt_Bat_SDM_R	GGAACACCCTAGAATGAAG	
Gr_Dog_Gly_F	TCCAAAGGGAACAAGACTTGC	64
Gr_Dog_Gly_R	TGCTCTCTTCCCTCTACTG	
A_Bat_Gly_F	GACGTGCGGGTTCGTTGACGAA	65
A_Bat_Gly_R	TTGTTTCCCTTCGATGCTTTCTTTCC	
CVS_Gly_F	ATCCAAAGGGGACAAGACTTG	61
CVS_Gly_R	GCTCTCTTCCCTCTGCTA	
EBLV_1_Gly_F	GACGTGTGGATTTGTGGATGAG	59
EBLV_1_Gly_R	TTGTTGTCTTTGGTTGCTTTCTTTC	
EBLV_2_Gly_F	TACAAAAGATAACAAAACGTGCG	56
EBLV_2_Gly_R	GCTTTCTTCCCCTTACTG	
	qPCR primers	
GAPDH_F	GACTTCAACAGTGACTCCCAC	60
GAPDH_R	CCTGTTGCTGTAGCCAAATTC	
HPRT1_F	GGACTAATTATGGACAGGACTG	60
HPRT1_R	GCTCTTCAGTCTGATAAAATCTAC	
nAChRa1_F	CACCTACCACTTCGTCATG	60
nAChRa1_R	ATGCTCAGTGTCATCTTCTC	
NCAM1_F	CACTCTTACCTGTGAAGCCT	60
NCAM1_R	CACCATGTGCCCATCCA	
qP75Fwd	CACAGTGATGGGCAGCTC	60
qP75Rev	GCTGGTCRTGCAGGCTCT	

Appendix II – Buffer recipes

Cell lysis buffer

50 mM Tris-HCl pH 7.4

150 mM NaCl

1 mM EDTA

1% IGEPAL

20 mM Iodoacetamide added on day of use

2x loading buffer (reducing)

120 mM Tris-HCl pH 6.8

200mM DTT

2x loading buffer (non-reducing)

120 mM Tris-HCl pH6.8

Transfer buffer

25 mM Tris Base

190 mM glycine

20% Methanol

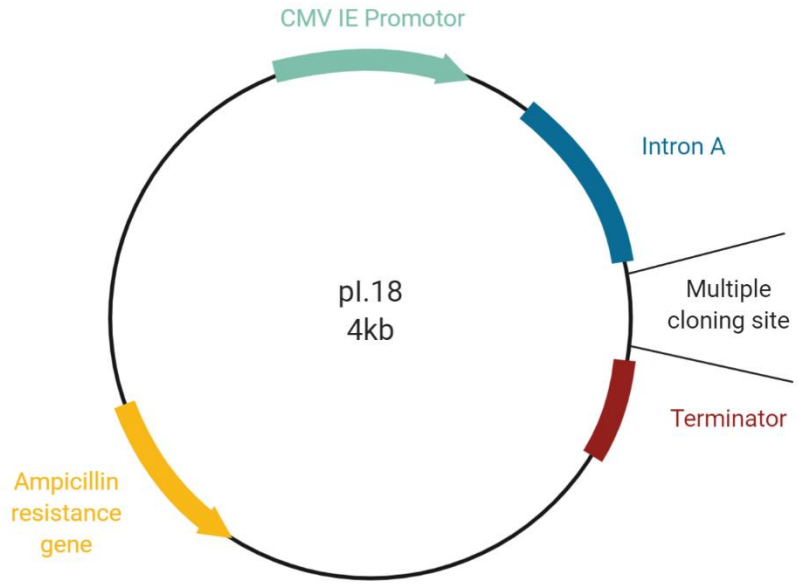
PBS-TWEEN

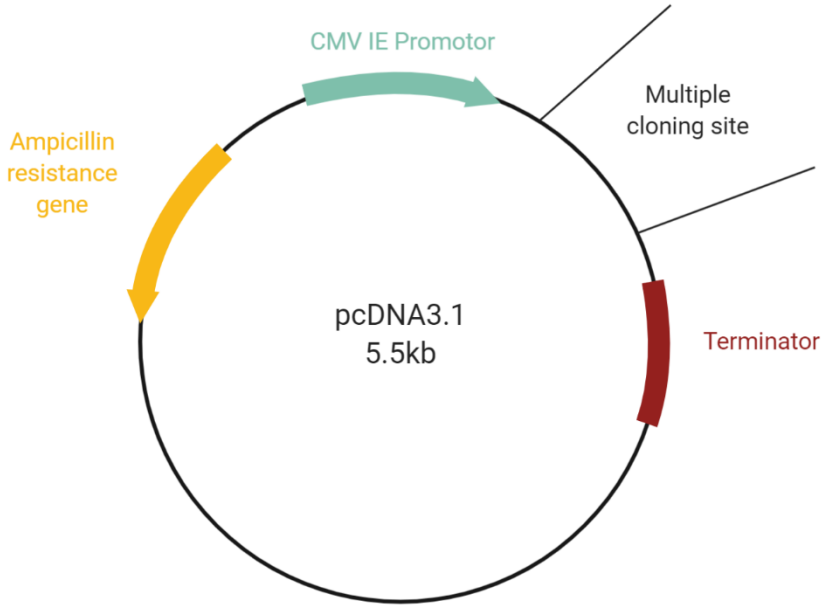
1x PBS

0.1% TWEEN

Appendix III – Plasmid maps

Simplified plasmid maps for pI.18 and pcDNA3.1.





Appendix IV – Internship reflective statement

As part of my PhD, I went on a three-month internship placement with Cellomatics Biosciences in Nottingham. They are a small contract research company that specialises in cell-based assays.

Heading into my internship, I hoped to experience what it was like to work within industry, as all my experience so far had been within academic labs, so I was curious to see how the day to day life differed.

Whilst on my internship I helped out with several projects for different clients, though the majority of my work was on one major project involving primary keratinocytes. I had assumed prior to starting the placement that the work undertaken would be fairly routine and that the company would spend most of the time performing standard, optimised assays for clients and that results would be obtained fairly quickly. I was surprised to discover that research in industry isn't so different from in academia, however with the added pressure of shortened timescales and the constant flow of additional projects.

As part of the major project I was assisting with, there were several major issues that needed to be overcome with the primary keratinocytes. These cells in particular were troublesome to grow and would have difficulty re-adhering to plasticware once they had been removed. Not only that, but they were fairly difficult to dissociate once they were attached, causing there to be a delicate balance between dissociation and causing large amounts of damage to the cells. Different dissociation reagents were tested; however the cells were consistently stubborn to remove. Plasticware was also coated with a collagen matrix to help encourage the cells to adhere, but there would always be a sizeable proportion of cells that remained in suspension. Whilst working with these cells, I noticed that the cells would typically adhere without issues after being defrosted from the liquid nitrogen, and issues with adherence only arose following dissociation. This was a helpful breakthrough, as it aided with setting up assays since cells could be bulked up to a reasonable extent, then frozen and plated directly into the appropriate plates for assays straight after being defrosted.

Due to the issues with cell adhesion, assay design was tricky as the cells could not proliferate when they were not adherent, therefore replicated could be inconsistent. Various methods of normalising data were attempted, such as through using cell titre kits to estimate the number of cells present in each well prior to collecting results, however some assays remained inconsistent.

Whilst working at Cellomatics, I got to experience working with some interesting technology that wasn't present in my PhD lab. The most useful of these was a live-cell imaging platform that sat inside an incubator, allowing cells to be monitored in real time. As this piece of kit was new to the lab at the time, there were various quirks that needed to be ironed out, but the utilities of the device were noticeable. Another technology that was new to me was a DNA hybridisation technology. This involved the use of magnetic beads with specific probe sets attached that could hybridise with target RNA sequences within a sample. Each probe set was associated with a different coloured bead, enabling multiple genes to be assessed at once for expression. This was an interesting method of detecting gene expression, though it had the downside of requiring reagents to be bought from a specific manufacturer and lacked the flexibility of more conventional techniques such as qPCR.

During my time on the internship, I also got the opportunity to experience more of the business side of industry and was involved in phone calls with clients and attempted producing a quotation for an incoming project.

My experience with Cellomatics gave me a good insight into working for a small company and I definitely learnt a lot. The most surprising outcome for me is that the internship showed me that a career in industry wasn't suitable for me, as previously I'd assumed that's where I'd end up after my PhD. It's difficult to pinpoint precisely where the issue lies, but a major factor is the intense troubleshooting that needed to be performed under very tight timescales. New projects were constantly in the pipeline, so there wasn't the opportunity to really dig into a project. Whilst this allowed for there to be some variety in the work that was performed, it also meant that there wasn't time to build up an in-

depth knowledge for the research being performed and as some of the projects were interesting this was a real shame.

Overall, I'm grateful for my time at Cellomatics, and whilst it did not lead to any clarity regarding my desired future career path, I feel my time there helped me grow as a person and my lab work in the last 8 months of my PhD benefited from my experiences.

References

1. Adamson PB. The spread of rabies into Europe and the probable origin of this disease in antiquity. J R Asiat Soc Gt Britain Ireland. 1977;109: 140–144. doi:10.1017/S0035869X00133829
2. Warrell MJ, Warrell DA. Rabies and other lyssavirus diseases. Lancet. 2004;363: 959–969. doi:10.1016/S0140-6736(04)15792-9
3. WHO. WHO Expert Consultation on Rabies: Second Report. World Health Organization Technical Report Series. 2013. doi:92 4 120931 3
4. Rupprecht C, Kuzmin I, Meslin F. Lyssaviruses and rabies: current conundrums, concerns, contradictions and controversies. F1000Research. 2017;6. doi:10.12688/f1000research.10416.1
5. Finnegan CJ, Brookes SM, Johnson N, Smith J, Mansfield KL, Keene VL, et al. Rabies in North America and Europe. J R Soc Med. 2002;95: 9–13. doi:10.1258/jrsm.95.1.9
6. Lembo T, Hampson K, Haydon DT, Craft M, Dobson A, Dushoff J, et al. Exploring reservoir dynamics: A case study of rabies in the Serengeti ecosystem. J Appl Ecol. 2008;45: 1246–1257. doi:10.1111/j.1365-2664.2008.01468.x
7. Escobar LE, Peterson AT, Favi M, Yung V, Medina-Vogel G. Bat-borne rabies in Latin America. Rev Inst Med Trop Sao Paulo. 2015;57: 63–72. doi:10.1590/S0036-46652015000100009
8. Rupprecht CE, Turmelle A, Kuzmin I V. A perspective on lyssavirus emergence and perpetuation. Curr Opin Virol. 2011;1: 662–670. doi:10.1016/j.coviro.2011.10.014
9. Nel LH. Discrepancies in data reporting for rabies, Africa. Emerg Infect Dis. 2013;19: 529–533. doi:10.3201eid1904.120185
10. Shope RE, Murphy FA, Harrison AK, Causey OR, Kemp GE, Simpson DIH, et al. Two African viruses serologically and morphologically related to rabies virus. J Virol. 1970;6: 690–692. Available: http://jvi.asm.org/content/6/5/690.abstract
11. Sabeta CT, Markotter W, Mohale DK, Shumba W, Wandeler AI, Nel LH. Mokola virus in domestic mammals, South Africa. Emerg Infect Dis.

2007;13: 1371–1373. doi:10.3201/eid1309.070466

12. Marston DA, Horton DL, Ngeleja C, Hampson K, McElhinney LM, Banyard AC, et al. Ikoma lyssavirus, highly divergent novel lyssavirus in an African civet. Emerg Infect Dis. 2012;18: 664–667. doi:10.3201/eid1804.111553
13. Banyard AC, Evans JS, Luo TR, Fooks AR. Lyssaviruses and bats: Emergence and zoonotic threat. Viruses. 2014;6: 2974–2990. doi:10.3390/v6082974
14. Tjørnehøj K, Fooks AR, Agerholm JS, Rønsholt L. Natural and Experimental Infection of Sheep with European Bat Lyssavirus Type-1 of Danish Bat Origin. J Comp Pathol. 2006;134: 190–201. doi:10.1016/j.jcpa.2005.10.005
15. Shinwari MW, Annand EJ, Driver L, Warrilow D, Harrower B, Allcock RJN, et al. Australian bat lyssavirus infection in two horses. Vet Microbiol. 2014;173: 224–231. doi:10.1016/j.vetmic.2014.07.029
16. Seganti L, Superti F, Bianchi S, Orsi N, Divizia M, Panà A, et al. Susceptibility of mammalian, avian, fish and mosquito cell lines to rabies virus infection. Acta Virol. 1990;34: 155–163.
17. Baby J, Mani RS, Abraham SS, Thankappan AT, Pillai PM, Anand AM, et al. Natural Rabies Infection in a Domestic Fowl (Gallus domesticus): A Report from India. PLoS Negl Trop Dis. 2015;9: e0003942. doi:10.1371/journal.pntd.0003942
18. Nel LH, Markotter W. Lyssaviruses. Crit Rev Microbiol. 2007;33: 301–324. doi:10.1080/10408410701647602
19. Weir DL, Smith IL, Bossart KN, Wang LF, Broder CC. Host cell tropism mediated by Australian bat lyssavirus envelope glycoproteins. Virology. 2013;444: 21–30. doi:10.1016/j.virol.2013.06.016
20. Thoulouze M-I, Lafage M, Schachner M, Hartmann U, Cremer H, Lafon M. The Neural Cell Adhesion Molecule Is a Receptor for Rabies Virus. J Virol. 1998;72: 7181–7190.
21. Lakhanpal U, Sharma RC. An epidemiological study of 177 cases of human rabies. Int J Epidemiol. 1985;14: 614–617. doi:10.1093/ije/14.4.614
22. Johnson N, Fooks A, McColl K. Reexamination of Human Rabies Case with Long Incubation, Australia. Emerg Infect Dis. 2008;14: 1950–1951.

doi:10.1093/ije/14.4.614

23. Smith JS, Fishbein DB, Rupprecht CE, Clark K. Unexplained rabies in three immigrants in the United States. N Engl J Med. 1991;324: 205–211.
24. Dato VM, Campagnolo ER, Long J, Rupprecht CE. A Systematic Review of Human Bat Rabies Virus Variant Cases: Evaluating Unprotected Physical Contact with Claws and Teeth in Support of Accurate Risk Assessments. PLoS One. 2016;11: e0159443. doi:10.1371/journal.pone.0159443
25. Chen S, Zhang H, Luo M, Chen J, Yao D, Chen F, et al. Rabies Virus Transmission in Solid Organ Transplantation, China, 2015–2016. Emerg Infect Dis. 2017;23: 1600–1602. doi:10.3201/eid2309.161704
26. Saeed B, Al-Mousawi M. Rabies Acquired Through Kidney Transplantation in a Child: A Case Report. Exp Clin Transplant. 2017;15: 355–357. doi:10.6002/ect.2017.0046
27. Vora NM, Basavaraju S V., Feldman KA, Paddock CD, Orciari L, Gitterman S, et al. Raccoon rabies virus variant transmission through solid organ transplantation. JAMA - J Am Med Assoc. 2013;310: 398–407. doi:10.1001/jama.2013.7986
28. Knobel DL, Cleaveland S, Coleman PG, Fèvre EM, Meltzer MI, Miranda MEG, et al. Re-evaluating the burden of rabies in Africa and Asia. Bull World Health Organ. 2003;008862: 360–369. doi:/S0042-96862005000500012
29. Hampson K, Coudeville L, Lembo T, Sambo M, Kieffer A, Attlan M, et al. Estimating the Global Burden of Endemic Canine Rabies. PLoS Negl Trop Dis. 2015;9: e0003709. doi:10.1371/journal.pntd.0003709
30. Meltzer MI, Rupprecht CE. A Review of the Economics of the Prevention and Control of Rabies. Part 1: Global Impact and Rabies in Humans. Pharmacoeconomics. 1998;14: 365–383.
31. Meltzer MI, Rupprecht CE. A Review of the Economics of the Prevention and Control of Rabies. Part 2: Rabies in Dogs, Livestock and Wildlife. Pharmacoeconomics. 1998;14: 365–383.
32. Fraser GC, Hooper PT, Lunt RA, Gould AR, Gleeson LJ, Hyatt AD, et al. Encephalitis Caused by a Lyssavirus in Fruit Bats in Australia. Emerg Infect Dis. 1996;2: 327–330. doi:10.3201/eid0204.960408

33. Hanna JN, Carney IK, Smith GA, Deverill JE, Botha JA, Serafin IL, et al. Australian bat lyssavirus infection: A second human case, with a long incubation period. Med J Aust. 2000;172: 597–599.
34. Allworth A, Murray K, Morgan J. A human case of encephalitis due to a lyssavirus recently identified in fruit bats. Commun Dis Intell. 1996;20: 504. Available: http://ezproxy.stir.ac.uk/login?url=http://search.ebscohost.com/login.aspx?direct=true&db=edsihc&AN=447067547202147&site=eds-live%5Cnhttp://search.informit.com.au/documentSummary%5Cndn=447067547202147%5Cnres=IELHEA
35. Weir DL, Annand EJ, Reid PA, Broder CC. Recent observations on Australian bat lyssavirus tropism and viral entry. Viruses. 2014;6: 909–926. doi:10.3390/v6020909
36. Brookes SM, Aegerter JN, Smith GC, Healy DM, Jolliffe TA, Swift SM, et al. European bat lyssavirus in Scottish bats. Emerg Infect Dis. 2005;11: 572–578. doi:10.3201/eid1104.040920
37. Nathwani D, McIntyre PG, White K, Shearer AJ, Reynolds N, Walker D, et al. Fatal human rabies caused by European bat Lyssavirus type 2a infection in Scotland. Clin Infect Dis. 2003;37: 598–601. doi:10.1086/376641
38. Eggerbauer E, Pfaff F, Finke S, Höper D, Beer M, Mettenleiter TC, et al. Comparative analysis of European bat lyssavirus 1 pathogenicity in the mouse model. PLoS Negl Trop Dis. 2017;11: e0005668. doi:10.1371/journal.pntd.0005668
39. UK Government PHE. First detection of European Bat Lyssavirus-1 in a serotine bat in the UK [Internet]. 2018 [cited 1 Feb 2020]. Available: https://www.england.nhs.uk/south/wp-content/uploads/sites/6/2018/11/briefing-note-bat-rabies-20181011.pdf
40. Hemachudha T, Laothamatas J, Rupprecht CE. Human rabies: A disease of complex neuropathogenetic mechanisms and diagnostic challenges. Lancet Neurol. 2002;1: 101–109. doi:10.1016/S1474-4422(02)00041-8
41. Schnell MJ, McGettigan JP, Wirblich C, Papaneri A. The cell biology of

rabies virus: using stealth to reach the brain. Nat Rev Microbiol. 2010;8: 51–61. doi:10.1038/nrmicro2260

42. Fooks AR, Banyard AC, Horton DL, Johnson N, McElhinney LM, Jackson AC. Current status of rabies and prospects for elimination. Lancet. 2014;384: 1389–1399. doi:10.1016/S0140-6736(13)62707-5
43. Wilde H. Failures of post-exposure rabies prophylaxis. Vaccine. 2007;25: 7605–7609. doi:10.1016/j.vaccine.2007.08.054
44. Lafon M, Bourhy H, Sureau P. Immunity against the European bat rabies (Duvenhage) virus induced by rabies vaccines: an experimental study in mice. Vaccine. 1988;6: 362–368. doi:10.1016/0264-410X(88)90184-3
45. Pearce JMS. Louis Pasteur and rabies: a brief note. J Neurol Neurosurg Psychiatry. 2002;73. doi:10.1136/jnnp.73.1.82
46. Wu X, Smith TG, Rupprecht CE. From brain passage to cell adaptation: The road of human rabies vaccine development. Expert Rev Vaccines. 2011;10: 1597–1608. doi:10.1586/erv.11.140
47. Ertl HCJ. New Rabies Vaccines for Use in Humans. Vaccines. 2019;7: 54. doi:10.3390/vaccines7020054
48. Astray RM, Jorge SAC, Pereira CA. Rabies vaccine development by expression of recombinant viral glycoprotein. Arch Virol. 2017;162: 323–332. doi:10.1007/s00705-016-3128-9
49. Fooks AR, Banyard AC, Ertl HCJ. New human rabies vaccines in the pipeline. Vaccine. 2019;37: A140–A145. doi:10.1016/j.vaccine.2018.08.039
50. Alberer M, Gnad-Vogt U, Hong HS, Mehr KT, Backert L, Finak G, et al. Safety and immunogenicity of a mRNA rabies vaccine in healthy adults: an open-label, non-randomised, prospective, first-in-human phase 1 clinical trial. Lancet. Elsevier Ltd; 2017;390: 1511–1520. doi:10.1016/S0140-6736(17)31665-3
51. Bourhy H, Bruckner L, Correa De Moura W, Ferguson M, Grachev V, Gibert R, et al. Recommendations for inactivated rabies vaccine for human use produced in cell substrates and embryonated eggs. World Health Organ Tech Rep Ser. 2007; 83–132.
52. WHO. Information Sheet, observed rate of vaccine reactions, rabies

vaccine. In: World Health Organization [Internet]. 2012 [cited 11 Mar 2020]. Available: https://www.who.int/vaccine_safety/initiative/tools/Rabies_Vaccine_rates_information_sheet.pdf

53. CDC. Principles of Vaccination. In: Hamborsky J, Kroger A, Wolfe C, editors. Epidemiology and prevention of vaccine-preventable diseases. 13th ed. Washington DC Public Health Foundation; 2015. pp. 263–278.
54. Cenna J, Hunter M, Tan GS, Papaneri AB, Ribka EP, Schnell MJ, et al. Replication-Deficient Rabies Virus–Based Vaccines Are Safe and Immunogenic in Mice and Nonhuman Primates. J Infect Dis. 2009;200: 1251–1260. doi:10.1086/605949
55. Hosokawa-Muto J, Ito N, Yamada K, Shimizu K, Sugiyama M, Minamoto N. Characterization of recombinant rabies virus carrying double glycoprotein genes. Microbiol Immunol. 2006;50: 187–196. doi:10.1111/j.1348-0421.2006.tb03785.x
56. Ertl HCJ. Novel vaccines to human rabies. PLoS Negl Trop Dis. 2009;3. doi:10.1371/journal.pntd.0000515
57. McKee AS, Marrack P. Old and new adjuvants. Curr Opin Immunol. 2017;47: 44–51. doi:10.1016/j.coi.2017.06.005
58. Del Giudice G, Rappuoli R, Didierlaurent AM. Correlates of adjuvanticity: A review on adjuvants in licensed vaccines. Semin Immunol. Elsevier; 2018;39: 14–21. doi:10.1016/j.smim.2018.05.001
59. Kalimuddin S, Wijaya L, Chan YFZ, Wong AWL, Oh HML, Wang LF, et al. A phase II randomized study to determine the safety and immunogenicity of the novel PIKA rabies vaccine containing the PIKA adjuvant using an accelerated regimen. Vaccine. The Author(s); 2017;35: 7127–7132. doi:10.1016/j.vaccine.2017.10.097
60. Doener F, Hong HS, Meyer I, Tadjalli-Mehr K, Daehling A, Heidenreich R, et al. RNA-based adjuvant CV8102 enhances the immunogenicity of a licensed rabies vaccine in a first-in-human trial. Vaccine. Elsevier Ltd; 2019;37: 1819–1826. doi:10.1016/j.vaccine.2019.02.024
61. Donovan D, Davies JC, Beath A, Stevenson B, Brown L, Sobey K, et al. Prevalence of Tetracycline and Rabies Virus Antibody in Raccoons,

Skunks, and Foxes Following Aerial Distribution of V-Rg Baits To Control Raccoon Rabies in Ontario, Canada. J Wildl Dis. 2013;44: 946–964. doi:10.7589/0090-3558-44.4.946

62. Knowles MK, Nadin-Davis SA, Sheen M, Rosatte R, Mueller R, Beresford A. Safety studies on an adenovirus recombinant vaccine for rabies (AdRG1.3-ONRAB®) in target and non-target species. Vaccine. 2009;27: 6619–6626. doi:10.1016/j.vaccine.2009.08.005
63. Morris SJ, Sebastian S, Spencer AJ, Gilbert SC. Simian adenoviruses as vaccine vectors. Future Virol. 2016;11: 649–659. doi:10.2217/fvl-2016-0070
64. Mähl P, Cliquet F, Guiot A-L, Niin E, Fournials E, Saint-Jean N, et al. Twenty year experience of the oral rabies vaccine SAG2 in wildlife: A global review. Vet Res. 2014;45. doi:10.1186/s13567-014-0077-8
65. Maki J, Guiot AL, Aubert M, Brochier B, Cliquet F, Hanlon CA, et al. Oral vaccination of wildlife using a vaccinia-rabies-glycoprotein recombinant virus vaccine (RABORAL V-RG®): A global review. Vet Res. BioMed Central; 2017;48: 1–26. doi:10.1186/s13567-017-0459-9
66. Bakker ABH, Python C, Kissling CJ, Pandya P, Marissen WE, Brink MF, et al. First administration to humans of a monoclonal antibody cocktail against rabies virus: Safety, tolerability, and neutralizing activity. Vaccine. 2008;26: 5922–5927. doi:10.1016/j.vaccine.2008.08.050
67. Wilde H, Khawplod P, Hemachudha T, Sitprija V. Postexposure Treatment of Rabies Infection: Can It Be Done without Immunoglobulin? Clin Infect Dis. 2002;34: 477–480. doi:10.1086/324628
68. Nagarajan T, Marissen WE, Rupprecht CE. Monoclonal antibodies for the prevention of rabies: theory and clinical practice. Antib Technol J. 2014;4: 1–12. doi:10.2147/ANTI.S33533
69. De Benedictis P, Minola A, Rota Nodari E, Aiello R, Zecchin B, Salomoni A, et al. Development of broad-spectrum human monoclonal antibodies for rabies post-exposure prophylaxis. EMBO Mol Med. 2016;8: 407–421. doi:10.15252/emmm.201505986
70. Gogtay NJ, Munshi R, Ashwath Narayana DH, Mahendra BJ, Kshirsagar V, Gunale B, et al. Comparison of a Novel Human Rabies Monoclonal

Antibody to Human Rabies Immunoglobulin for Postexposure Prophylaxis: A Phase 2/3, Randomized, Single-Blind, Noninferiority, Controlled Study. Clin Infect Dis. 2018;66: 387–395. doi:10.1093/cid/cix791

71. Sparrow E, Torvaldsen S, Newall AT, Wood JG, Sheikh M, Kieny MP, et al. Recent advances in the development of monoclonal antibodies for rabies post exposure prophylaxis: A review of the current status of the clinical development pipeline. Vaccine. The Authors; 2019;37: A132–A139. doi:10.1016/j.vaccine.2018.11.004
72. Chao T-YY, Ren S, Shen E, Moore S, Zhang SF, Chen L, et al. SYN023, a novel humanized monoclonal antibody cocktail, for post-exposure prophylaxis of rabies. PLoS Negl Trop Dis. 2017;11: e0006133. doi:10.1371/journal.pntd.0006133
73. Hanlon CA, Kuzmin I V., Blanton JD, Weldon WC, Manangan JS, Rupprecht CE. Efficacy of rabies biologics against new lyssaviruses from Eurasia. Virus Res. 2005;111: 44–54. doi:10.1016/j.virusres.2005.03.009
74. Johnson N, Vos A, Freuling C, Tordo N, Fooks AR, Müller T. Human rabies due to lyssavirus infection of bat origin. Vet Microbiol. 2010;142: 151–159. doi:10.1016/j.vetmic.2010.02.001
75. Evans JS, Horton DL, Easton AJ, Fooks AR, Banyard AC. Rabies virus vaccines: Is there a need for a pan-lyssavirus vaccine? Vaccine. 2012;30: 7447–7454. doi:10.1016/j.vaccine.2012.10.015
76. Wallace RM, Undurraga EA, Blanton JD, Cleaton J, Franka R. Elimination of dog-mediated human rabies deaths by 2030: needs assessment and alternatives for progress based on dog vaccination. Front Vet Sci. 2017;4. doi:10.3389/fvets.2017.00009
77. WHO. Global elimination of dog-mediated human rabies. Rep Rabies Glob Conf. 2015; Available: http://apps.who.int/iris/bitstream/10665/204621/1/WHO_HTM_NTD_NZD_2016.02_eng.pdf?ua=1
78. Stoner-Duncan B, Streicker DG, Tedeschi CM. Vampire Bats and Rabies: Toward an Ecological Solution to a Public Health Problem. PLoS Negl Trop Dis. 2014;8: 8–12. doi:10.1371/journal.pntd.0002867

79. Willoughby RE, Tieves KS, Hoffman GM, Ghanayem NS, Amlie-Lefond CM, Schwabe MJ, et al. Survival after treatment of rabies with induction of coma. N Engl J Med. 2005;352: 2508–2514. doi:10.1056/NEJMoa050382
80. Zeiler FA, Jackson AC. Critical Appraisal of the Milwaukee Protocol for Rabies: This Failed Approach Should Be Abandoned. Can J Neurol Sci. 2016;43: 44–51. doi:10.1017/cjn.2015.331
81. Ferrara EA, Oishi JS, Wannemacher RW, Stephen EL. Plasma disappearance, urine excretion, and tissue distribution of ribavirin in rats and rhesus monkeys. Antimicrob Agents Chemother. 1981;19: 1042–1049. doi:10.1128/AAC.19.6.1042
82. Appolinario CM, Jackson AC. Antiviral therapy for human rabies. Antivir Ther. 2015;20: 1–10. doi:10.3851/IMP2851
83. Appolinário CM, Prehaud C, Allendorf SD, Azevedo JM, Peres MG, Lafon M, et al. Ribavirin has an in vitro antiviral effect in rabies virus infected neuronal cells but fails to provide benefit in experimental rabies in mice. J Virol Antivir Res. 2013;02. doi:10.4172/2324-8955.1000111
84. Kureishi A, Xu LZ, Stiver HG. Rabies in China: Recommendations for control. Bull World Health Organ. 1992;70: 443–450.
85. Lockhart BP, Tsiang H, Ceccaldi PE, Guillemer S. Ketamine-mediated inhibition of rabies virus infection in vitro and in rat brain. Antivir Chem Chemother. 1991;2: 9–15. doi:10.1177/095632029100200102
86. Bussereau F, Chermann JC, De Clercq E, Hannoun C. Search for compounds which have an inhibitory effect on rhabdovirus multiplication in vitro. Ann l'Institute Pasteur/Virologie. 1983;134: 127–134.
87. Gilbert AT, Petersen BW, Recuenco S, Niezgoda M, Gómez J, Laguna-Torres VA, et al. Evidence of rabies virus exposure among humans in the Peruvian Amazon. Am J Trop Med Hyg. 2012;87: 206–215. doi:10.4269/ajtmh.2012.11-0689
88. Morimoto K, Patel M, Corisdeo S, Hooper DC, Fu ZF, Rupprecht CE, et al. Characterization of a unique variant of bat rabies virus responsible for newly emerging human cases in North America. Proc Natl Acad Sci. 1996;93: 5653–5658. doi:10.1073/pnas.93.11.5653
89. Badrane H, Bahloul C, Perrin P, Tordo N. Evidence of two Lyssavirus

phylogroups with distinct pathogenicity and immunogenicity. J Virol. 2001;75: 3268–3276. doi:10.1128/JVI.75.7.3268

90. Horton DL, Banyard AC, Marston DA, Wise E, Selden D, Nunez A, et al. Antigenic and genetic characterization of a divergent African virus, Ikoma lyssavirus. J Gen Virol. 2014;95: 1025–1032. doi:10.1099/vir.0.061952-0
91. Ceballos NA, Morón SV, Berciano JM, Nicolás O, López CA, Juste J, et al. Novel Lyssavirus in Bat, Spain. Emerg Infect Dis. 2013;19: 793–795. doi:http://dx.doi.org/10.3201/eid1905.121071
92. Schneider LG, Dietzschold B, Dierks RE, Matthaeus W, Enzmann PJ, Strohmaier K. Rabies group specific ribonucleoprotein antigen and a test system for grouping and typing of rhabdoviruses. J Virol. 1973;11: 748–755.
93. Bourhy H, Kissi B, Tordo N. Molecular Diversity of the Lyssavirus Genus. Virology. 1993;194: 70–81.
94. McElhinney LM, Marston DA, Leech S, Freuling CM, van der Poel WHM, Echevarria J, et al. Molecular Epidemiology of Bat Lyssaviruses in Europe. Zoonoses Public Health. 2013;60: 35–45. doi:10.1111/zph.12003
95. Velasco-Villa A, Orciari LA, Juárez-Islas V, Gómez-Sierra M, Padilla-Medina I, Flisser A, et al. Molecular diversity of rabies viruses associated with bats in Mexico and other countries of the Americas. J Clin Microbiol. 2006;44: 1697–1710. doi:10.1128/JCM.44.5.1697-1710.2006
96. Van Zyl N, Markotter W, Nel LH. Evolutionary history of African mongoose rabies. Virus Res. 2010;150: 93–102. doi:10.1016/j.virusres.2010.02.018
97. Troupin C, Dacheux L, Tanguy M, Sabeta C, Blanc H, Bouchier C, et al. Large-Scale Phylogenomic Analysis Reveals the Complex Evolutionary History of Rabies Virus in Multiple Carnivore Hosts. PLoS Pathog. 2016;12: e1006041. doi:http://dx.doi.org/10.1371/journal.ppat.1006041
98. Guyatt KJ, Twin J, Davis P, Holmes EC, Smith GA, Mackenzie JS, et al. A molecular epidemiological study of Australian bat lyssavirus. J Gen Virol. 2003;84: 485–496. doi:10.1099/vir.0.18652-0
99. Finke S, Cox JH, Conzelmann K-K. Differential transcription attenuation of rabies virus genes by intergenic regions: generation of recombinant viruses overexpressing the polymerase gene. J Virol. 2000;74: 7261–

7269. doi:10.1128/JVI.74.16.7261-7269.2000

100. Faber M, Pulmanausahakul R, Hodawadekar SS, Spitsin S, McGettigan JP, Schnell MJ, et al. Overexpression of the Rabies Virus Glycoprotein Results in Enhancement of Apoptosis and Antiviral Immune Response. J Virol. 2002;76: 3374–3381. doi:10.1128/JVI.76.7.3374
101. Yang J, Koprowski H, Dietzschold B, Fu ZF. Phosphorylation of rabies virus nucleoprotein regulates viral RNA transcription and replication by modulating leader RNA encapsidation. J Virol. 1999;73: 1661–1664.
102. Kissi B, Tordo N, Bourhy H. Genetic Polymorphism in the Rabies Virus Nucleoprotein Gene. Virology. 1995;209: 526–537. doi:10.1006/viro.1995.1285
103. Liu P, Yang J, Wu X, Fu ZF. Interactions amongst rabies virus nucleoprotein, phosphoprotein and genomic RNA in virus-infected and transfected cells. J Gen Virol. 2004;85: 3725–3734. doi:10.1099/vir.0.80325-0
104. Wunner WH, Larson JK, Dietzschold B, Smith CL. The molecular biology of rabies viruses. Rev Infect Dis. 1988;10: S771–S784. doi:10.1093/clinids/10.Supplement_4.S771
105. Masatani T, Ito N, Shimizu K, Ito Y, Nakagawa K, Sawaki Y, et al. Rabies Virus Nucleoprotein Functions To Evade Activation of the RIG-I-Mediated Antiviral Response. J Virol. 2010;84: 4002–4012. doi:10.1128/jvi.02220-09
106. Yang J, Hooper DC, Wunner WH, Koprowski H, Dietzschold B, Fu ZF. The specificity of rabies virus RNA encapsidation by nucleoprotein. Virology. 1998;242: 107–117. doi:10.1006/viro.1997.9022
107. Li Y, Dong W, Shi Y, Deng F, Chen X, Wan C, et al. Rabies virus phosphoprotein interacts with ribosomal protein L9 and affects rabies virus replication. Virology. Elsevier; 2016;488: 216–224. doi:10.1016/j.virol.2015.11.018
108. Vidy A, Chelbi-Alix M, Blondel D. Rabies Virus P Protein Interacts with STAT1 and Inhibits Interferon Signal Transduction Pathways. J Virol. 2005;79: 14411–14420. doi:10.1128/jvi.79.22.14411-14420.2005
109. Mebatsion T, Weiland F, Conzelmann K-K. Matrix Protein of Rabies Virus

Is Responsible for the Assembly and Budding of Bullet-Shaped Particles and Interacts with the Transmembrane Spike Glycoprotein G. J Virol. 1999;73: 242–250. doi:10.1128/JVI.00554-09

110. Hummeler K, Koprowski H, Wiktor TJ. Structure and development of rabies virus in tissue culture. J Virol. 1967;1: 152–70. Available: http://www.ncbi.nlm.nih.gov/pubmed/4918232%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC375516
111. Coll JM. The glycoprotein G of rhabdoviruses. Arch Virol. 1995;140: 827–851. doi:10.1007/BF01314961
112. Delmas O, Holmes EC, Talbi C, Larrous F, Dacheux L, Bouchier C, et al. Genomic diversity and evolution of the lyssaviruses. PLoS One. 2008;3: e2057. doi:10.1371/journal.pone.0002057
113. Ogino M, Ito N, Sugiyama M, Ogino T. The rabies virus L protein catalyzes mRNA capping with GDP polyribonucleotidyltransferase activity. Viruses. 2016;8: 1–10. doi:10.3390/v8050144
114. Liu W, Liu Y, Liu J, Zhai J, Xie Y. Evidence for inter- and intra-clade recombinations in rabies virus. Infect Genet Evol. 2011;11: 1906–1912. doi:10.1016/j.meegid.2011.08.031
115. Gaudin Y. Folding of rabies virus glycoprotein: epitope acquisition and interaction with endoplasmic reticulum chaperones. J Virol. 1997;71: 3742–3750.
116. Anilionis A, Wunner WH, Curtis PJ. Structure of the glycoprotein gene in rabies virus. Nature. 1981;294: 275–278. doi:10.1038/294275a0
117. Martoglio B, Dobberstein B. Signal sequences: More than just greasy peptides. Trends Cell Biol. 1998;8: 410–415. doi:10.1016/S0962-8924(98)01360-9
118. Marissen WE, Kramer RA, Rice A, Weldon WC, Niezgoda M, Faber M, et al. Novel Rabies Virus-Neutralizing Epitope Recognized by Human Monoclonal Antibody: Fine Mapping and Escape Mutant Analysis. J Virol. 2005;79: 4672–4678. doi:10.1128/JVI.79.8.4672-4678.2005
119. Fernando B-G, Yersin C-T, José C-B, Paola Z-S. Predicted 3D Model of the Rabies Virus Glycoprotein Trimer. Biomed Res Int. 2016;2016: 1674580. doi:10.1155/2016/1674580

120. Roche S, Rey FA, Gaudin Y, Bressanelli S. Structure of the Prefusion Form of VSV G. Science (80-). 2007;315: 843–848. doi:10.1126/science.1135710
121. Roche S, Bressanelli S, Rey FA, Gaudin Y. Crystal structure of the low-pH form of the vesicular stomatitis virus glycoprotein G. Science (80-). 2006;313: 187–191. doi:10.1126/science.1127683
122. Buthelezi SG, Dirr HW, Chakauya E, Chikwamba R, Martens L, Tsekoa TL, et al. The Lyssavirus glycoprotein: A key to cross-immunity. Virology. 2016;498: 250–256. doi:10.1016/j.virol.2016.08.034
123. Walker PJ, Kongsuwan K. Deduced structural model for animal rhabdovirus glycoproteins. J Gen Virol. 1999;80: 1211–1220. doi:10.1099/0022-1317-80-5-1211
124. Gaudin Y, Ruigrok RWH, Tuffereau C, Knossow M, Flamand A. Rabies virus glycoprotein is a trimer. Virology. 1992;187: 627–632.
125. Piccinotti S, Whelan SPJ. Rabies internalizes into primary peripheral neurons via clathrin coated pits and requires fusion at the cell body. PLoS Pathog. 2016;12: e1005753. doi:10.1371/journal.ppat.1005753
126. Gaudin Y, Tuffereau C, Segretain D, Knossow M, Flamand A. Reversible Conformational Changes and Fusion Activity of Rabies Virus Glycoprotein. J Virol. 1991;65: 4853–4859.
127. White JM, Delos SE, Brecher M, Schornberg K. Structures and Mechanisms of Viral Membrane Fusion Proteins. Crit Rev Biochem Mol Biol. 2008;43: 189–219. doi:10.1080/10409230802058320.Structures
128. Vigerust DJ, Shepherd VL. Virus glycosylation: role in virulence and immune interactions. Trends Microbiol. 2007;15: 211–218. doi:10.1016/j.tim.2007.03.003
129. Shakin-Eshleman SH, Remaley AT, Eshleman JR, Wunner WH, Spitalnik SL. N-Linked Glycosylation of Rabies Virus Glycoprotein. J Biol Chem. 1992;267: 10690–10698.
130. Koch M, Pancera M, Kwong PD, Kolchinsky P, Grundner C, Wang L, et al. Structure-based, targeted deglycosylation of HIV-1 gp120 and effects on neutralization sensitivity and antibody recognition. Virology. 2003;313: 387–400. doi:10.1016/S0042-6822(03)00294-0

131. Shakin-Eshleman SH, Spitalnik SL, Kasturi L. The Amino Acid at the X Position of an Asn-X-Ser Sequon Is an Important Determinant of N-Linked Core-glycosylation Efficiency. J Biol Chem. 1996;271: 6363–6366. doi:10.1074/JBC.271.11.6363
132. Kasturi L, Eshleman JR, Wunner WH, Shakin-Eshleman SH. The Hydroxy Amino Acid in an Asn- X -Ser/Thr Sequon Can Influence N -Linked Core Glycosylation Efficiency and the Level of Expression of a Cell Surface Glycoprotein [Internet]. Journal of Biological Chemistry. 1995. pp. 14756–14761. doi:10.1074/jbc.270.24.14756
133. Irie T, Matsuda Y, Honda Y, Morimoto K, Kawai A. Studies on the escape mutants of rabies virus which are resistant to neutralization by a highly conserved conformational epitope-specific monoclonal antibody #1-46-12. Microbiol Immunol. 2002;46: 449–461. doi:10.1111/j.1348-0421.2002.tb02719.x
134. Rose JK, Doolittle RF, Anilionis A, Curtis PJ, Wunner WH. Homology between the glycoproteins of vesicular stomatitis virus and rabies virus. J Virol. 1982;43: 361–364.
135. Wojczyk BS, Stwora-Wojczyk M, Shakin-Eshleman S, Wunner WH, Spitalnik SL. The role of site-specific N-glycosylation in secretion of soluble forms of rabies virus glycoprotein. Glycobiology. 1998;8: 121–130. doi:10.1093/glycob/8.2.121
136. Wunner WH, Dietzschold B, Smith CL, Lafon M, Golub E. Antigenic variants of CVS rabies virus with altered glycosylation sites. Virology. 1985;140: 1–12. doi:10.1016/0042-6822(85)90440-4
137. Benmansour A, Leblois H, Coulon P, Tuffereau C, Gaudin Y, Flamand A, et al. Antigenicity of rabies virus glycoprotein. J Virol. 1991;65: 4198–4203.
138. Lafon M, Wiktor TJ, Macfarlan RI. Antigenic Sites on the CVS Rabies Virus Glycoprotein : Analysis with Monoclonal Antibodies. J Gen Virol. 1983;64: 843–851.
139. Prehaud C, Coulon P, Lafay F, Thiers C, Flamand A. Antigenic site II of the rabies virus glycoprotein: structure and role in viral virulence. J Virol. 1988;62: 1–7. doi:10.1016/j.virusres.2005.04.004
140. Evans JS, Selden D, Wu G, Wright E, Horton DL, Fooks AR, et al. Antigenic

site changes in the rabies virus glycoprotein dictates functionality and neutralizing capability against divergent lyssaviruses. J Gen Virol. 2018;99: 1–12. doi:10.1099/jgv.0.000998

141. Dietzschold B, Wunner WH, Wiuror TJ, Lopes AD, Lafon M, Smith CL, et al. Characterization of an antigenic determinant of the glycoprotein that correlates with pathogenicity of rabies virus (monoclonal antibody/peptide mapping/protein sequence analysis/DNA sequence analysis). Biochemistry. 1983;80: 70–74. doi:10.1073/pnas.80.1.70
142. Seif I, Coulon P, Rollin PE, Flamand A. Rabies virulence: effect on pathogenicity and sequence characterization of rabies virus mutations affecting antigenic site III of the glycoprotein. J Virol. 1985;53: 926–34. Available: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=254728&tool=pmcentrez&rendertype=abstract
143. Desmaris N, Bosch A, Petit C, Perrin P, Schwartz O, Rocquigny H De, et al. Production and Neurotropism of Lentivirus Vectors Pseudotyped with Lyssavirus Envelope Glycoproteins. Mol Ther. 2001;4: 149–156. doi:10.1006/mthe.2001.0431
144. Lentz TL, Burrage TG, Smith AL, Crick J, Tignor GH. Is the acetylcholine receptor a rabies virus receptor? Science (80-). 1982;215: 182–184. doi:10.1126/science.7053569
145. Albuquerque EX, Pereira EFR, Alkondon M, Rogers SW. Mammalian nicotinic acetylcholine receptors: from structure to function. Physiol Rev. 2009;89: 73–120. doi:10.1152/physrev.00015.2008.Mammalian
146. Millar NS, Gotti C. Diversity of vertebrate nicotinic acetylcholine receptors. Neuropharmacology. Elsevier Ltd; 2009;56: 237–246. doi:10.1016/j.neuropharm.2008.07.041
147. Millar NS. Assembly and subunit diversity of nicotinic acetylcholine receptors. Biochem Soc Trans. 2003;31: 869–874. doi:10.1042/BST0310869
148. Lentz TL. Rabies virus binding to an acetylcholine receptor alpha-subunit peptide. J Mol Recognit. 1990;3: 82–88. doi:10.1002/jmr.300030205
149. Lentz TL, Wilson PT, Hawrot E, Speicher DW. Amino acid similarity

between Rabies Virus glycoprotein and snake venom curaremimetic neurotoxins. Science (80-). 1984;226: 847–848.

150. Hanham CA, Zhao F, Tignor GH. Evidence from the anti-idiotypic network that the acetylcholine receptor is a rabies virus receptor. J Virol. 1993;67: 530–542.
151. Lafon M. Rabies virus receptors. J Neurovirol. 2005;11: 82–87. doi:10.1016/0166-2236(85)90123-7
152. Hueffer K, Khatri S, Rideout S, Harris MB, Papke RL, Stokes C, et al. Rabies virus modifies host behaviour through a snake-toxin like region of its glycoprotein that inhibits neurotransmitter receptors in the CNS. Sci Rep. 2017;7: 12818. doi:10.1038/s41598-017-12726-4
153. Reagan KJ, Wunner WH. Rabies virus interaction with various cell lines is independent of the acetylcholine receptor. Arch Virol. 1985;84: 277–282. doi:10.1007/BF01378980
154. Covault J, Sanes JR. Distribution of N-CAM in synaptic and extrasynaptic portions of developing and adult skeletal muscle. J Cell Biol. 1986;102: 716–730. doi:10.1083/jcb.102.3.716
155. Polo-Parada L, Bose CM, Plattner F, Landmesser LT. Distinct Roles of Different Neural Cell Adhesion Molecule (NCAM) Isoforms in Synaptic Maturation Revealed by Analysis of NCAM 180 kDa Isoform-Deficient Mice. J Neurosci. 2004;24: 1852–1864. doi:10.1523/JNEUROSCI.4406-03.2004
156. Weledji EP, Assob JC. The ubiquitous neural cell adhesion molecule (N-CAM). Ann Med Surg. 2014;3: 77–81. doi:10.1016/j.amsu.2014.06.014
157. Hotta K, Motoi Y, Okutani A, Kaku Y, Noguchi A, Inoue S, et al. Role of GPI-anchored NCAM-120 in rabies virus infection. Microbes Infect. 2007;9: 167–174. doi:10.1016/j.micinf.2006.11.003
158. Meeker RB, Williams KS. The p75 neurotrophin receptor: At the crossroad of neural repair and death. Neural Regen Res. 2015;10: 721–725. doi:10.4103/1673-5374.156967
159. Sheard PW, Musaad K, Duxson MJ. Distribution of neurotrophin receptors in the mouse neuromuscular system. Int J Dev Biol. 2002;46: 569–75.
160. Tuffereau C, Bénéjean J, Blondel D, Kieffer B, Flamand A. Low-affinity

nerve-growth factor receptor (P75NTR) can serve as a receptor for rabies virus. EMBO J. 1998;17: 7250–7259. doi:10.1093/emboj/17.24.7250

161. Langevin C, Jaaro H, Bressanelli S, Fainzilber M, Tuffereau C. Rabies virus glycoprotein (RVG) is a trimeric ligand for the N-terminal cysteine-rich domain of the mammalian p75 neurotrophin receptor. J Biol Chem. 2002;277: 37655–37662. doi:10.1074/jbc.M201374200
162. Tuffereau C, Schmidt K, Langevin C, Lafay F, Dechant G, Koltzenburg M. The rabies virus glycoprotein receptor p75NTR is not essential for rabies virus infection. J Virol. 2007;81: 13622–13630. doi:10.1128/JVI.02368-06
163. Wang J, Wang Z, Liu R, Shuai L, Wang X, Luo J, et al. Metabotropic glutamate receptor subtype 2 is a cellular receptor for rabies virus. PLoS Pathog. 2018;14: 1–21. doi:10.1371/journal.ppat.1007189
164. Moussawi K, Kalivas PW. Group II metabotropic glutamate receptors (mGlu2/3) in drug addiction. Eur J Pharmacol. Elsevier B.V.; 2010;639: 115–122. doi:10.1016/j.ejphar.2010.01.030
165. Superti F, Hauttecoeur B, Morelec M-J, Goldoni P, Bizzini B, Tsiang H. Involvement of gangliosides in rabies virus infection. J Gen Virol. 1986;67: 47–56.
166. Sasaki M, Anindita PD, Ito N, Sugiyama M, Carr M, Fukuhara H, et al. The role of heparan sulfate proteoglycans as an attachment factor for rabies virus entry and infection. J Infect Dis. 2018;217: 1740–1749. doi:10.1093/infdis/jiy081
167. Page KA, Landau NR, Littman DR. Construction and use of a human immunodeficiency virus vector for analysis of virus infectivity. J Virol. 1990;64: 5270–6. Available: http://www.ncbi.nlm.nih.gov/pubmed/2214018%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC248565
168. Naldini L, Trono D, Verma IM. Lentiviral vectors, two decades later. Science (80-). 2016;353: 1101–1102. doi:10.1126/science.aah6192
169. Temperton NJ, Wright E, Scott SD. Retroviral Pseudotypes – from scientific tools to clinical utility. Encycl Life Sci. 2015; 1–21. doi:10.1002/9780470015902.a0022548
170. King B, Temperton NJ, Grehan K, Scott SD, Wright E, Tarr AW, et al.

Technical considerations for the generation of novel pseudotyped viruses. Future Virol. 2016;11: 47–59.

171. Whitt MA. Generation of VSV pseudotypes using recombinant ΔG-VSV for studies on virus entry, identification of entry inhibitors, and immune responses to vaccines. J Virol Methods. Elsevier B.V.; 2010;169: 365–374. doi:10.1016/j.jviromet.2010.08.006
172. Bilska M, Tang H, Montefiori DC. Potential Risk of Replication-Competent Virus in HIV-1 Env-Pseudotyped Virus Preparations. AIDS Res Hum Retroviruses. 2017;33: 368–372. doi:10.1089/AID.2016.0142
173. Goila-Gaur R, Strebel K. HIV-1 Vif, APOBEC, and intrinsic immunity. Retrovirology. 2008;5: 1–16. doi:10.1186/1742-4690-5-51
174. Zufferey R, Nagy D, Mandel RJ, Naldini L, Trono D. Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. Nat Biotechnol. 1997;15: 871–875. doi:10.1038/nbt0997-871
175. Kogan M, Rappaport J. HIV-1 Accessory Protein Vpr: Relevance in the pathogenesis of HIV and potential for therapeutic intervention. Retrovirology. BioMed Central Ltd; 2011;8: 25. doi:10.1186/1742-4690-8-25
176. Dubé M, Bego MG, Paquay C, Cohen ÉA. Modulation of HIV-1-host interaction: Role of the Vpu accessory protein. Retrovirology. BioMed Central Ltd; 2010;7: 114. doi:10.1186/1742-4690-7-114
177. Luo T, Douglas JL, Livingston RL, Garcia JV. Infectivity enhancement by HIV-1 Nef is dependent on the pathway of virus entry: Implications for HIV-based gene transfer systems. Virology. 1998;241: 224–233. doi:10.1006/viro.1997.8966
178. Basmaciogullari S, Pizzato M. The activity of Nef on HIV-1 infectivity. Front Microbiol. 2014;5: 1–12. doi:10.3389/fmicb.2014.00232
179. Sandrin V, Cosset FL. Intracellular versus cell surface assembly of retroviral pseudotypes is determined by the cellular localization of the viral glycoprotein, its capacity to interact with Gag, and the expression of the Nef protein. J Biol Chem. 2006;281: 528–542. doi:10.1074/jbc.M506070200
180. Wright E, Temperton NJ, Marston DA, McElhinney LM, Fooks AR, Weiss

RA. Investigating antibody neutralization of lyssaviruses using lentiviral pseudotypes: A cross-species comparison. J Gen Virol. 2008;89: 2204–2213. doi:10.1099/vir.0.2008/000349-0

181. Valtink M, Stanke N, Knels L, Engelmann K, Funk RHW, Lindemann D. Pseudotyping and culture conditions affect efficiency and cytotoxicity of retroviral gene transfer to human corneal endothelial cells. Invest Ophthalmol Vis Sci. 2011;52: 6807–13. doi:10.1167/iovs.11-7710
182. Urbanowicz RA, McClure CP, King B, Mason CP, Ball JK, Tarr AW. Novel functional hepatitis C virus glycoprotein isolates identified using an optimised viral pseudotype entry assay. J Gen Virol. 2016;97: 2265–2279. doi:10.1099/jgv.0.000537
183. Kobinger GP, Weiner DJ, Yu QC, Wilson JM. Filovirus-pseudotyped lentiviral vector can efficiently and stably transduce airway epithelia in vivo. Nat Biotechnol. 2001;19: 225–230. doi:10.1038/85664
184. Urbanowicz RA, McClure CP, Sakuntabhai A, Sall AA, Kobinger G, Müller MA, et al. Human Adaptation of Ebola Virus during the West African Outbreak. Cell. 2016;167: 1079–1087. doi:10.1016/j.cell.2016.10.013
185. Mohan GS, Ye L, Li W, Monteiro A, Lin X, Sapkota B, et al. Less is more: Ebola virus surface glycoprotein expression levels regulate virus production and infectivity. J Virol. 2015;89: 1205–17. doi:10.1128/JVI.01810-14
186. Shedlock DJ, Bailey MA, Popernack PM, Cunningham JM, Burton DR, Sullivan NJ. Antibody-mediated neutralization of Ebola virus can occur by two distinct mechanisms. Virology. 2010;401: 228–235. doi:10.1016/j.virol.2010.02.029
187. Wright E, Hayman DTS, Vaughan A, Temperton NJ, Wood JLN, Cunningham AA, et al. Virus neutralising activity of African fruit bat (Eidolon helvum) sera against emerging lyssaviruses. Virology. Elsevier Inc.; 2010;408: 183–189. doi:10.1016/j.virol.2010.09.014
188. Wilkinson DE, Page M, Mattiuzzo G, Hassall M, Dougall T, Rigsby P, et al. Comparison of platform technologies for assaying antibody to Ebola virus. Vaccine. The Author(s); 2017;35: 1347–1352. doi:10.1016/j.vaccine.2016.11.083

189. Sacramento D, Badrane H, Bourhy H, Tordo N. Molecular epidemiology of rabies virus in France: Comparison with vaccine strains. J Gen Virol. 1992;73: 1149–1158. doi:10.1099/0022-1317-73-5-1149
190. Marston DA, McElhinney LM, Johnson N, Müller T, Conzelmann KK, Tordo N, et al. Comparative analysis of the full genome sequence of European bat lyssavirus type 1 and type 2 with other lyssaviruses and evidence for a conserved transcription termination and polyadenylation motif in the G-L 3' non-translated region. J Gen Virol. 2007;88: 1302–1314. doi:10.1099/vir.0.82692-0
191. Marston DA, McElhinney LM, Ellis RJ, Horton DL, Wise EL, Leech SL, et al. Next generation sequencing of viral RNA genomes. BMC Genomics. 2013;14. doi:10.1186/1471-2164-14-444
192. Markotter W, Kuzmin I, Rupprecht CE, Nel LH. Phylogeny of Lagos bat virus: Challenges for lyssavirus taxonomy. Virus Res. 2008;135: 10–21. doi:10.1016/j.virusres.2008.02.001
193. Horton DL, McElhinney LM, Marston DA, Wood JLN, Russell CA, Lewis N, et al. Quantifying antigenic relationships among the lyssaviruses. J Virol. 2010;84: 11841–11848. doi:10.1128/JVI.01153-10
194. Kuzmin I V., Mayer AE, Niezgoda M, Markotter W, Agwanda B, Breiman RF, et al. Shimoni bat virus, a new representative of the Lyssavirus genus. Virus Res. 2010;149: 197–210. doi:10.1016/j.virusres.2010.01.018
195. Kuzmin I V., Wu X, Tordo N, Rupprecht CE. Complete genomes of Aravan, Khujand, Irkut and West Caucasian bat viruses, with special attention to the polymerase gene and non-coding regions. Virus Res. 2008;136: 81–90. doi:10.1016/j.virusres.2008.04.021
196. Marston DA, Ellis RJ, Horton DL, Kuzmin I V, Wise EL, McElhinney LM, et al. Complete genome sequence of Ikoma lyssavirus. J Virol. 2012;86: 10242–10243. doi:10.1128/JVI.01628-12
197. Wright E, McNabb S, Goddard T, Horton DL, Lembo T, Nel LH, et al. A robust lentiviral pseudotype neutralisation assay for in-field serosurveillance of rabies and lyssaviruses in Africa. Vaccine. 2009;27: 7178–7186. doi:10.1016/j.vaccine.2009.09.024
198. Hanke D, Freuling CM, Fischer S, Hueffer K, Hundertmark K, Nadin-Davis

S, et al. Spatio-temporal Analysis of the Genetic Diversity of Arctic Rabies Viruses and Their Reservoir Hosts in Greenland. PLoS Negl Trop Dis. 2016;10: e0004779. doi:10.1371/journal.pntd.0004779

199. Kuzmin I V., Niezgoda M, Franka R, Agwanda B, Markotter W, Beagley JC, et al. Lagos bat virus in Kenya. J Clin Microbiol. 2008;46: 1451–1461. doi:10.1128/JCM.00016-08
200. Zhang L, Cui Z, Zhou L, Kang Y, Li L, Li J, et al. Developing a triple transgenic cell line for high-efficiency porcine reproductive and respiratory syndrome virus infection. PLoS One. 2016;11: e0154238. doi:10.1371/journal.pone.0160325
201. Valadan R, Hedayatizadeh-Omran A, Alhosseini-Abyazani MN, Amjadi O, Rafiei A, Tehrani M, et al. Data supporting the design and evaluation of a universal primer pair for pseudogene-free amplification of HPRT1 in real-time PCR. Data Br. 2015;4: 384–389. doi:10.1016/j.dib.2015.06.009
202. Kuzmin I V., Novella IS, Dietzgen RG, Padhi A, Rupprecht CE. The rhabdoviruses: Biodiversity, phylogenetics, and evolution. Infect Genet Evol. 2009;9: 541–553. doi:10.1016/j.meegid.2009.02.005
203. Paweska JT, Blumberg LH, Liebenberg C, Hewlett RH, Grobbelaar AA, Leman PA, et al. Fatal human infection with rabies-related Duvenhage virus, South Africa. Emerg Infect Dis. 2006;12: 1965–1967. doi:10.3201/eid1212.060764
204. Meslin FX, Kaplan MM, Koprowski H, WHO. Laboratory Techniques in Rabies. 4th ed. World Health Organization. 1996. doi:10.1136/jcp.49.11.955-b
205. King B, Daly J. Pseudotypes : your flexible friends. Future Microbiol. 2014;9: 135–137. doi:10.2217/fmb.13.156
206. Mentis GZ, Gravell M, Hamilton R, Shneider NA, O'Donovan MJ, Schubert M. Transduction of motor neurons and muscle fibers by intramuscular injection of HIV-1-based vectors pseudotyped with select rabies virus glycoproteins. J Neurosci Methods. 2006;157: 208–217. doi:10.1016/j.jneumeth.2006.04.011
207. Carpentier DCJ, Vevis K, Trabalza A, Georgiadis C, Ellison SM, Asfahani RI, et al. Enhanced pseudotyping efficiency of HIV-1 lentiviral vectors by a

rabies/vesicular stomatitis virus chimeric envelope glycoprotein. Gene Ther. 2012;19: 761–774. doi:10.1038/gt.2011.124

208. Nie J, Wu X, Ma J, Cao S, Huang W, Liu Q, et al. Development of in vitro and in vivo rabies virus neutralization assays based on a high-titer pseudovirus system. Sci Rep. 2017;7: 42769. doi:10.1038/srep42769
209. Federici T, Kutner R, Zhang X-Y, Kuroda H, Tordo N, Boulis NM, et al. Comparative analysis of HIV-1-based lentiviral vectors bearing lyssavirus glycoproteins for neuronal gene transfer. Genet Vaccines Ther. 2009;7. doi:10.1186/1479-0556-7-1
210. Todd D, Connor TJ, Creelan JL, Borghmans BJ, Calvert VM, McNulty MS. Effect of multiple cell culture passages on the biological behaviour of chicken anaemia virus. Avian Pathol. 1998;27: 74–79. doi:10.1080/03079459808419277
211. Kuzmina NA, Kuzmin I V., Ellison JA, Rupprecht CE. Conservation of binding epitopes for Monoclonal Antibodies on the rabies virus glycoprotein. J Antivirals Antiretrovir. 2013;5: 037–043. doi:10.4172/jaa.1000061
212. Yap MW, Colbeck E, Ellis SA, Stoye JP. Evolution of the Retroviral Restriction Gene Fv1: Inhibition of Non-MLV Retroviruses. PLoS Pathog. 2014;10: e1003968. doi:10.1371/journal.ppat.1003968
213. Ellison JA, Gilbert AT, Recuenco S, Moran D, Alvarez DA, Kuzmina N, et al. Bat Rabies in Guatemala. PLoS Negl Trop Dis. 2014;8: e3070. doi:10.1371/journal.pntd.0003070
214. Streicker DG, Altizer SM, Velasco-Villa A, Rupprecht CE. Variable evolutionary routes to host establishment across repeated rabies virus host shifts among bats. Proc Natl Acad Sci. 2012;109: 19715–19720. doi:10.1073/pnas.1203456109
215. Holmes EC, Woelk CH, Kassis R, Bourhy H. Genetic constraints and the adaptive evolution of rabies virus in nature. Virology. 2002;292: 247–257. doi:10.1006/viro.2001.1271
216. Marston DA, Horton DL, Nunez J, Ellis RJ, Orton RJ, Johnson N, et al. Genetic analysis of a rabies virus host shift event reveals within-host viral dynamics in a new host. Virus Evol. 2017;3: vex038.

doi:10.1093/ve/vex038

217. Kissi B, Badrane H, Audry L, Lavenu A, Tordo N, Brahimi M, et al. Dynamics of rabies virus quasispecies during serial passages in heterologous hosts. J Gen Virol. 1999;80: 2041–2050. doi:10.1099/0022-1317-80-8-2041
218. Alazard-Dany N, Volchkova V, Reynard O, Carbonnelle C, Dolnik O, Ottmann M, et al. Ebola virus glycoprotein GP is not cytotoxic when expressed constitutively at a moderate level. J Gen Virol. 2006;87: 1247–1257. doi:10.1099/vir.0.81361-0
219. Temperton NJ, Hoschler K, Major D, Nicolson C, Manvell R, Hien VM, et al. A sensitive retroviral pseudotype assay for influenza H5N1-neutralizing antibodies. Influenza Other Respi Viruses. 2007;1: 105–112. doi:10.1111/j.1750-2659.2007.00016.x
220. Quilici LS, Silva-Pereira I, Andrade AC, Albuquerque FC, Brigido MM, Maranhão AQ. A minimal cytomegalovirus intron A variant can improve transgene expression in different mammalian cell lines. Biotechnol Lett. 2013;35: 21–27. doi:10.1007/s10529-012-1043-z
221. Kang M, Kim S-Y, Lee S, Lee Y-K, Lee J, Shin H-S, et al. Human β-globin second intron highly enhances expression of foreign genes from murine cytomegalovirus immediate-early promoter. J Microbiol Biotechnol. 2005;15: 544–550.
222. Li C, Zhang F, Lin H, Wang ZC, Liu XJ, Feng ZQ, et al. Generation and characterization of the human neutralizing antibody fragment Fab091 against rabies virus. Acta Pharmacol Sin. Nature Publishing Group; 2011;32: 329–337. doi:10.1038/aps.2010.209
223. Kutner RH, Zhang X-Y, Reiser J. Production, concentration and titration of pseudotyped HIV-1-based lentiviral vectors. Nat Protoc. 2009;4: 495–505. doi:10.1038/nprot.2009.22
224. Sastry L, Johnson T, Hobson MJ, Smucker B, Cornetta K. Titering lentiviral vectors: Comparison of DNA, RNA and marker expression methods. Gene Ther. 2002;9: 1155–1162. doi:10.1038/sj.gt.3301731
225. Ge P, Tsao J, Schein S, Green TJ, Luo M, Zhou ZH. Cryo-EM model of the bullet-shaped vesicular stomatitis virus. Science (80-). 2010;327: 689–

693. doi:10.1126/science.1181766

226. Bentley EM, Ali R, Horton DL, Corti D, Banyard AC, Fooks AR, et al. Generation of Arctic-like Rabies Viruses Containing Chimeric Glycoproteins Enables Serological Potency Studies. bioRxiv. 2017; doi:10.1101/150300

227. Malerczyk C, Freuling C, Gniel D, Giesen A, Selhorst T, Müller T. Cross-neutralization of antibodies induced by vaccination with Purified Chick Embryo Cell Vaccine (PCECV) against different Lyssavirus species. Hum Vaccines Immunother. 2014;10: 2799–2804. doi:10.4161/21645515.2014.972741

228. Brookes SM, Parsons G, Johnson N, McElhinney LM, Fooks AR. Rabies human diploid cell vaccine elicits cross-neutralising and cross-protecting immune responses against European and Australian bat lyssaviruses. Vaccine. 2005;23: 4101–4109. doi:10.1016/j.vaccine.2005.03.037

229. von Teichman BF, de Koker WC, Bosch SJE, Bishop GC, Meredith CD, Bingham J. Mokola virus infection: description of recent South African cases and a review of the virus epidemiology. J S Afr Vet Assoc. 1998;69: 169–171. doi:10.4102/jsava.v69i4.847

230. Cliquet F, Aubert M, Sagné L. Development of a fluorescent antibody virus neutralisation test (FAVN test) for the quantitation of rabies-neutralising antibody. J Immunol Methods. 1998;212: 79–87. doi:10.1016/S0022-1759(97)00212-3

231. Moore SM, Hanlon CA. Rabies-specific antibodies: Measuring surrogates of protection against a fatal disease. PLoS Negl Trop Dis. 2010;4: e595. doi:10.1371/journal.pntd.0000595

232. Briggs DJ, Smith JS, Mueller FL, Schwenke J, Davis RD, Gordon CR, et al. A comparison of two serological methods for detecting the immune response after rabies vaccination in dogs and cats being exported to rabies-free areas. Biologicals. 1998;26: 347–355. doi:10.1006/biol.1998.0162

233. Abe Y, Takashita E, Sugawara K, Matsuzaki Y, Muraki Y, Hongo S. Effect of the Addition of Oligosaccharides on the Biological Activities and Antigenicity of Influenza A / H3N2 Virus Hemagglutinin. J Virol. 2004;78:

9605–9611. doi:10.1128/JVI.78.18.9605

234. WHO. Rabies vaccines: WHO position paper April 2018. Wkly Epidemiol Rec. 2018;16: 201–220. doi:10.1016/j.actatropica.2012.04.013.

235. Swaddiwuthipong W, Weniger BG, Wattanasri S, Warrell MJ. A high rate of neurological complications following semple anti-rabies vaccine. Trans R Soc Trop Med Hyg. 1988;82: 472–475. doi:10.1016/0035-9203(88)90167-8

236. Hampson K, Dobson A, Kaare M, Dushoff J, Magoto M, Sindoya E, et al. Rabies exposures, post-exposure prophylaxis and deaths in a region of endemic canine rabies. PLoS Negl Trop Dis. 2008;2. doi:10.1371/journal.pntd.0000339

237. Wu Q, Yu F, Xu J, Li Y, Chen H, Xiao S, et al. Rabies-virus-glycoprotein-pseudotyped recombinant baculovirus vaccine confers complete protection against lethal rabies virus challenge in a mouse model. Vet Microbiol. Elsevier B.V.; 2014;171: 93–101. doi:10.1016/j.vetmic.2014.03.037

238. Nakagawa KKKKKK, Nakagawa KKKKKK, Omatsu T, Katayama Y, Oba M, Mitake H, et al. Generation of a novel live rabies vaccine strain with a high level of safety by introducing attenuating mutations in the nucleoprotein and glycoprotein. Vaccine. Elsevier Ltd; 2017;35: 5622–5628. doi:10.1016/j.vaccine.2017.08.050

239. Alemany R, Rajan A, Custers J, Majhen D, Fajardo CA, Chandra N, et al. Adenovirus-Based Vaccines for Fighting Infectious Diseases and Cancer: Progress in the Field. Hum Gene Ther. 2014;25: 301–317. doi:10.1089/hum.2013.235

240. Fougeroux C, Holst PJ. Future prospects for the development of cost-effective adenovirus vaccines. Int J Mol Sci. 2017;18. doi:10.3390/ijms18040686

241. Xiang Z, Li Y, Cun A, Yang W, Ellenberg S, Switzer WM, et al. Chimpanzee adenovirus antibodies in humans, sub-Saharan Africa. Emerg Infect Dis. 2006;12: 1596–1599. doi:10.3201/eid1210.060078

242. Ersching J, Hernandez MIM, Cezarotto FS, Ferreira JDS, Martins AB, Switzer WM, et al. Neutralizing antibodies to human and simian

adenoviruses in humans and New-World monkeys. Virology. Elsevier Inc.; 2010;407: 1–6. doi:10.1016/j.virol.2010.07.043

243. Hirsch RL. The complement system: Its importance in the host response to viral infection. Microbiol Rev. 1982;46: 71–85. doi:10.1128/mmbr.46.1.71-85.1982

244. Cocchi F, Devico AL, Garzino-Demo A, Arya SK, Gallo RC, Lusso P. Identification the Major HIV-Suppressive Factors Produced by CD8+ T Cells. Science (80-). 1995;270: 1811–1815.

245. Brown KS, Keogh MJ, Owsianka AM, Adair R, Patel AH, Arnold JN, et al. Specific interaction of hepatitis C virus glycoproteins with mannan binding lectin inhibits virus entry. Protein Cell. 2010;1: 664–674. doi:10.1007/s13238-010-0088-9

246. Van Breedam W, Pöhlmann S, Favoreel HW, de Groot RJ, Nauwynck HJ. Bitter-sweet symphony: Glycan-lectin interactions in virus biology. FEMS Microbiol Rev. 2014;38: 598–632. doi:10.1111/1574-6976.12052

247. Rosatte RC, Donovan D, Davies JC, Allan M, Bachmann P, Stevenson B, et al. Aerial distribution of ONRAB baits as a tactic to control rabies in raccoons and striped skunks in Ontario, Canada. J Wildl Dis. 2009;45: 363–374. doi:10.7589/0090-3558-45.2.363

248. Kim H-H, Yang D-K, Nah J-J, Song J-Y, Cho I-S. Comparison of the protective efficacy between single and combination of recombinant adenoviruses expressing complete and truncated glycoprotein, and nucleoprotein of the pathogenic street rabies virus in mice. Virol J. 2017;14: 122. doi:10.1186/s12985-017-0789-2

249. Wang X, Fang Z, Xiong J, Yang K, Chi Y, Tang X, et al. A chimpanzee adenoviral vector-based rabies vaccine protects beagle dogs from lethal rabies virus challenge. Virology. 2019;536: 32–38. doi:10.1016/j.virol.2019.07.022

250. McFadden G. Poxvirus tropism. Nat Rev Microbiol. 2005;3: 201–213. doi:10.1038/nrmicro1099

251. Ma W, Kahn RE, Richt JA. The pig as a mixing vessel for influenza viruses: Human and veterinary implications. J Mol Genet Med. 2009;3: 158–166. doi:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2702078/

252. Rupprecht CE, Hanlon CA, Hemachudha T. Rabies re-examined. Lancet Infect Dis. 2002;2: 327–343. doi:10.1016/S1473-3099(02)00287-6
253. Fooks AR, Brookes SM, Johnson N, McElhinney LM, Hutson AM. European bat lyssaviruses: an emerging zoonosis. Epidemiol Infect. 2003;131: 1029–1039. doi:10.1017/S0950268803001481
254. Müller T, Cox J, Peter W, Schäfer R, Johnson N, McElhinney LM, et al. Spill-over of European bat lyssavirus type 1 into a stone marten (Martes foina) in Germany. J Vet Med Ser B Infect Dis Vet Public Heal. 2004;51: 49–54. doi:10.1111/j.1439-0450.2003.00725.x
255. Kgaladi J, Wright N, Coertse J, Markotter W, Marston D, Fooks AR, et al. Diversity and Epidemiology of Mokola Virus. PLoS Negl Trop Dis. 2013;7: e2511. doi:10.1371/journal.pntd.0002511
256. Picard-Meyer E, Barrat J, Wasniewski M, Wandeller A, Nadin-Davis S, Lowings JP, et al. Epidemiology of rabid bats in France, 1989 to 2002. Vet Rec. 2004;155: 774–777. doi:10.1136/vr.155.24.774
257. Moreira-Soto A, Soto-Garita C, Corrales-Aguilar E. Neotropical primary bat cell lines show restricted dengue virus replication. Comp Immunol Microbiol Infect Dis. Elsevier Ltd; 2017;50: 101–105. doi:10.1016/j.cimid.2016.12.004
258. Dheda K, Huggett JF, Bustin SA, Johnson MA, Rook G, Zumla A. Validation of housekeeping genes for normalizing RNA expression in real-time PCR. Biotechniques. 2004;37: 112–119. doi:https://doi.org/10.2144/04371RR03
259. Hayman DTS, Fooks AR, Rowcliffe JM, McCrea R, Restif O, Baker KS, et al. Endemic Lagos bat virus infection in Eidolon helvum. Epidemiol Infect. 2012;140: 2163–2171. doi:10.1017/S0950268812000167
260. Boulger LR, Porterfield JS. Isolation of a virus from Nigerian fruit bats. Trans R Soc Trop Med Hyg. 1958;52: 421–424. doi:10.1016/0035-9203(58)90127-5
261. Kgaladi J, Nel LH, Markotter W. Comparison of pathogenic domains of rabies and African rabies-related lyssaviruses and pathogenicity observed in mice. Onderstepoort J Vet Res. 2010;80: 511. doi:10.4102/ojvr.v80i1.511

262. Ikeda Y, Collins MKL, Radcliffe PA, Mitrophanous KA, Takeuchi Y. Gene transduction efficiency in cells of different species by HIV and EIAV vectors. Gene Ther. 2002;9: 932–938. doi:10.1038/sj.gt.3301708
263. Simmons G, Reeves JD, Rennekamp AJ, Amberg SM, Piefer AJ, Bates P. Characterization of severe acute respiratory syndrome-associated coronavirus (SARS-CoV) spike glycoprotein-mediated viral entry. Proc Natl Acad Sci. 2004;101: 4240–4245. doi:10.1073/pnas.0306446101
264. Besnier C, Ylinen L, Strange B, Lister A, Takeuchi Y, Goff SP, et al. Characterization of murine leukemia virus restriction in mammals. J Virol. 2003;77: 13403–13406. doi:10.1128/JVI.77.24.13403
265. Kozera B, Rapacz M. Reference genes in real-time PCR. J Appl Genet. 2013;54: 391–406. doi:10.1007/s13353-013-0173-x
266. Porteus M. Using homologous recombination to manipulate the genome of human somatic cells. Biotechnol Genet Eng Rev. 2007;24: 195–212. doi:10.1080/02648725.2007.10648100
267. Santiago Y, Chan E, Liu P-Q, Orlando S, Zhang L, Urnov FD, et al. Targeted gene knockout in mammalian cells by using engineered zinc-finger nucleases. Proc Natl Acad Sci. 2008;105: 5809–5814. doi:10.1073/pnas.0800940105
268. Boettcher M, McManus MT. Choosing the Right Tool for the Job: RNAi, TALEN, or CRISPR. Mol Cell. Elsevier Inc.; 2015;58: 575–585. doi:10.1016/j.molcel.2015.04.028
269. Housden BE, Perrimon N. Comparing CRISPR and RNAi-based screening technologies. Nat Biotechnol. Nature Publishing Group; 2016;34: 621–623. doi:10.1038/nbt.3599
270. Kumar M, Keller B, Makalou N, Sutton RE. Systematic determination of the packaging limit of lentiviral vectors. Hum Gene Ther. 2001;12: 1893–1905. doi:10.1089/104303401753153947
271. Shipley R, Wright E, Selden D, Wu G, Aegerter J, Fooks AR, et al. Bats and Viruses: Emergence of Novel Lyssaviruses and Association of Bats with Viral Zoonoses in the EU. Trop Med Infect Dis. 2019;4: 31. doi:10.3390/tropicalmed4010031
272. Jounai N, Okuda K, Kojima Y, Toda Y, Hamajima K, Ohba K, et al.

Contribution of the rev gene to the immunogenicity of DNA vaccines targeting the envelope glycoprotein of HIV. J Gene Med. 2003;5: 609–617. doi:10.1002/jgm.391

273. Nie Y, Wang P, Shi X, Wang G, Chen J, Zheng A, et al. Highly infectious SARS-CoV pseudotyped virus reveals the cell tropism and its correlation with receptor expression. Biochem Biophys Res Commun. 2004;321: 994–1000. doi:10.1016/j.bbrc.2004.07.060
274. Ramakrishnan MA. Determination of 50% endpoint titer using a simple formula. World J Virol. 2016;5: 85–86. doi:10.5501/wjv.v5.i2.85
275. LaBarre DD, Lowy RJ. Improvements in methods for calculating virus titer estimates from TCID50 and plaque assays. J Virol Methods. 2001;96: 107–126. doi:10.1016/S0166-0934(01)00316-0