Development of lentiviral pseudotypes for surveillance studies on animal influenza viruses

By REBECCA KINSLEY

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Medway School of Pharmacy
Universities of Kent and Greenwich at Medway
Central Avenue, Chatham, Kent, ME4 4TB

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DECLARATION

I certify that this work has not been accepted in substance for any degree, and is not concurrently being submitted for any degree other than that of the Doctor of Philosophy being studied at the Universities of Greenwich and Kent. I also declare that this work is the result of my own investigations except where otherwise identified by references and that I have not plagiarised the work of others.

PhD Student _ Rebecca Kinsley

_ Date

PhD Supervisor _ Simon Scott

_ Date

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ABSTRACT

Pseudotyped viruses (PVs) provide a safe, flexible platform for fundamental virological studies and antibody screening assays. Generation of influenza PVs involves co-transfection of producer cells with plasmids encoding the necessary viral components. The pseudotype virus neutralisation assay (PVNA) is a sensitive technique to measure protective antibody responses which cause neutralisation of virus particles. Many traditional methodologies, e.g. Haemagglutination Inhibition (HI) and Single Radial Haemolysis (SRH), detect only surface glycoprotein binding antibodies whereas the PVNA quantifies infectivity-neutralising responses.

Haemagglutinin (HA) and neuraminidase (NA) are the two major surface glycoproteins embedded within the membrane of an influenza virion. HA is responsible for virion attachment and entry into a host cell and NA is essential for viral egress and thus spread of infection. Two enzymes crucial for the infectivity of influenza viruses are; HA-cleaving cellular proteases and the NA itself. Optimisation of both enzymes in PV production is necessary to increase the titre of PVs. Producing high titre PVs is important as this permits minimal quantities to be used in PVNAs, and repeat experiments can be carried out using the same batch of virus, minimising intra-study variability.

Optimising PVs and employing them in a novel situation, such as an equine influenza vaccine efficacy trial, has been carried out with promising results. We have demonstrated that data obtained from the PVNA correlates well with the traditional SRH assay and consequently there is the potential for more widespread adoption in research and commercial settings. Furthermore, PVs have been manipulated to assess how single amino acid changes with equine influenza virus can affect the neutralisation efficacy of sera generated by vaccination.

Novel PVs, such as those derived from canine and phocine (seal) influenza strains have also been produced and provide a new platform for sero-surveillance of these viruses particularly in the case of wild or feral animals, due to issues with obtaining samples from wild animals during acute infection.

Overall, PVs have been demonstrated as useful and readily-manipulated tools for studying antibody responses against equine, canine and phocine influenza viruses.

CONTENTS

ABBREVIATIONS

Animal Health Trust	AHT
A/equine/Newmarket/1/1979	Nm79
A/equine/Sussex/1989	Sx89
A/equine/Newmarket/1/93	Nm/1/93
A/equine/Newmarket/5/03	Nm/5/03
A/equine/Richmond/1/07	Rich07
Canine influenza	CI
Committee for Proprietary Medicinal Products	CPMP
Cytopathic effect	CPE
Double distilled	dd
European Medicines Agency	EMA
Equine Influenza	EI
Equine-raised serum	E
Exogenous neuraminidase	ExNA
Ferret-raised serum	F
Forward (primer)	FWD
Haemagglutinin	HA
Haemagglutination inhibition assay	HI
Human embryonic kidney	HEK
Human airway trypsin-like protease	HAT
Kallikrein 5	KLK5
Kilobase pairs	Kb
Luria Bertani	LB
Multiple cloning site	MCS
Neuraminidase	NA
Polyethylenimine	PEI
Polymerase Chain Reaction	PCR
Pseudotype virus	PV
Pseudotype virus neutralisation assay	PVNA

Relative luminescence unit	RLU
Resolutions per minute	RPM
Restriction enzyme	RE
Reverse (primer)	REV
Site directed mutagenesis	SDM
Single radial haemolysis	SRH
Transmembrane serine protease	TMPRSS(2/3/4/6)
Volume/volume ratio	v/v
Weight/volume ratio	w/v
Wild-type	WT
World Organisation of Animal Health	OIE

FIGURES

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1 INTRODUCTION

1.1 Influenza virus

1.1.1 Classification

Influenza, the disease caused by the influenza virus, is a global concern for humans and many other animal species. There are four genera of influenza viruses belonging to the *Orthomyxoviridae* family; A, B, C and D. Influenza A virus is responsible for most morbidity and mortality amongst humans due to seasonal and pandemic outbreaks. It is also capable of causing global pandemics in humans (Chang *et al.*, 2009; Viboud *et al.*, 2005) and widespread outbreaks in birds (Alexander, 2000; Guan and Smith, 2013), pigs (Smith *et al.*, 2009), horses (Newton *et al.*, 2006), dogs (Yoon *et al.*, 2005), and seals (Anthony *et al.*, 2012). Influenza B virus currently circulates amongst humans but due to a slower rate of evolution they are a less likely pandemic threat. Humans and seals are the only known influenza B hosts (Osterhaus, 2000). Influenza C virus causes very mild disease in humans, pigs and dogs (Speranskaya *et al.*, 2012). Influenza D virus is the most recent addition to the *Orthomyxoviridae* family, isolated from pigs and cattle in 2011. Cattle workers have been shown to be seropositive for anti-influenza D antibodies but were not symptomatic for influenza (White *et al.*, 2016). Since the first isolation in pigs, the animal host range has extended to sheep and goats (Quast *et al.*, 2015).

1.1.2 Influenza A virion and genome structure

The influenza virion encapsulates a single stranded negative sense RNA genome that must first be transcribed into positive sense RNA before the genes can be translated into viral proteins. The genome consists of eight individual segments of viral RNA (vRNA), some of which code for multiple proteins. The pleomorphic, enveloped virion is studded with two main surface glycoproteins; haemagglutinin (**HA**) and neuraminidase (**NA**), which are expressed in approximately a 4:1 ratio respectively (Webster et al., 1968; Figure 1). Cryoelectron tomography images suggest that there are between 300 – 400 HA spikes per virion (Harris *et al.*, 2006). Embedded within the envelope are ion channels formed by the **M2** protein (matrix protein 2) and underlying the lipid envelope is matrix protein 1 (**M1**). The two M proteins are coded by one vRNA segment. The segments are

tightly arranged by the attraction of positively charged nucleoprotein (**NP**) to the negatively charged phosphate backbone of the vRNA. The virus encodes a heterotrimeric RNA polymerase complex; polymerase basic proteins (**PB1** & **PB2**) and polymerase acidic protein (**PA**), which associates with each of the eight vRNA segments, enabling transcription and replication of the virus genome. PB2 binds the 5' capped host pre-mRNA that is then cleaved by PA, essentially creating a primer from which PB1 synthesises mRNA (reviewed in te Velthuis and Fodor, 2016). The combination of vRNA segments, NP and trimeric-polymerase group form vRNP complexes that associate with M1 beneath the lipid envelope (Eisfeld *et al.*, 2014). Non-structural protein 1 (**NS1**) and nuclear export protein (**NEP** or sometimes referred to as NS2) are encoded on the same vRNA segment. The influenza genome was originally thought to encode ten proteins (highlighted in bold text above) but in 2001, the proteome was extended to 11 proteins and since then a further six proteins have been discovered (reviewed in Vasin et al., 2014; Table 1).

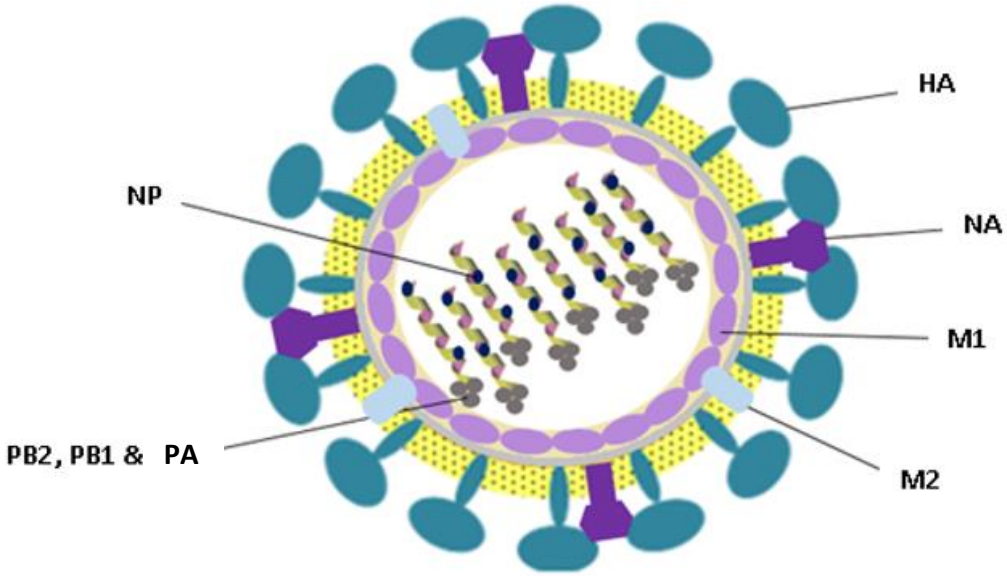


Figure 1. Structure of an influenza virion.

Table 1. Summary of influenza viral RNA (vRNA) segments, the proteins coded by each vRNA segment and their primary function (modified from Vasin et al., 2014).

vRNA segment	Viral Protein	Function
1	Polymerase basic protein 2 (PB2)	Polymerase subunit required for initiation of transcription via binding of 5′ capped host pre-mRNA (Guilligay *et al.*, 2008)
2	Polymerase basic protein 1 (PB1)	Polymerase subunit responsible for elongation of RNA
	PB1-F2	Virulence factor associated with death of host immune cells (Chen *et al.*, 2001)
	PB1-N40	Regulates expression of PB1 (Wise *et al.*, 2009)
3	Polymerase acidic protein (PA)	Polymerase subunit with RNA endonuclease activity (cap-snatching mechanism) (Dias *et al.*, 2009)
	PA-X	Alters host cell function via degradation of host cell mRNA (Jagger *et al.*, 2012)
	PA-N155	Role in virus replication but not yet fully understood (Muramoto *et al.*, 2013)
	PA-N182	
4	Haemagglutinin (HA)	Surface glycoprotein responsible for virus attachment to host cell sialic acid receptors, the major viral antigen and target of neutralising antibodies
5	Nucleoprotein (NP)	Import of vRNA into host cell nucleus and compact arrangement of vRNPs
6	Neuraminidase (NA)	Surface glycoprotein responsible for cleavage of sialic acid receptors enabling virus release
7	Matrix protein 1 (M1)	Interaction with vRNPs and export of vRNA from host cell nucleus
	Matrix protein 2 (M2)	Ion channel responsible for release of vRNA from endosome
	M42	Alternative M2 protein (Wise *et al.*, 2012)
8	Non-structural protein 1 (NS1)	Interferon antagonist
	Nuclear export protein (NEP)	Export of vRNA from host cell nucleus
	NS3	Possible link to adaptation in novel hosts (Selman *et al.*, 2012)

1.1.3 Nomenclature

The two major surface glycoproteins (HA and NA) are used as the basis for influenza virus nomenclature e.g. H5N1. At present there are 18 HA and 11 NA known subtypes (Centers for Disease Control and Prevention, 2016). Influenza strains are recorded as shown in Figure 2. Human influenza strains follow the same nomenclature pattern as other species however the host species is omitted.

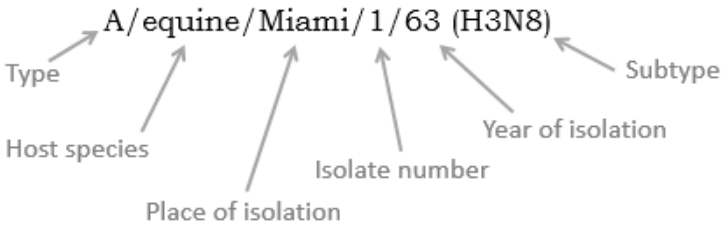


Figure 2. Example of influenza virus nomenclature.

1.1.4 Influenza virus life cycle

Attachment of an influenza virion to a host cell depends on the HA surface glycoprotein binding to sialic (N-acetylneuraminic) acid receptors. The receptors are abundant on many different cell types found across a variety of animal species. Two different linkages can be formed between the carbon backbone of the terminal sialic acid and galactose, in a 2,3 or 2,6 arrangement, forming α-2,3 or α-2,6 linkages (Bouvier & Palese, 2008). The HA protein recognises the different linkages and preferentially binds to one type.

A prerequisite to successful infection is cleavage of the HA precursor, HA0, into HA1 and HA2 subunits by specific cellular proteases (Figure 3). Cleavage occurs subcellularly within the trans-Golgi network but can also take place at the cell surface (Garten *et al.*, 2015). Once the HA is bound to the surface of the cell, the virion is endocytosed into an endosome. The acidic pH within the endosome triggers a conformational change in the HA trimeric-structured protein. The change in protein structure is crucial for exposure of a fusion peptide, facilitating the fusion of the HA with the endosomal membrane, which creates a pore for the vRNPs to escape into the cell cytoplasm (Stegmann, 2000). The M2 ion channel reduces the pH within the virion, as

hydrogen ions are pumped in from the endosome, encouraging the dissociation of M1 and aiding the release of genetic material (Martin & Helenius, 1991).

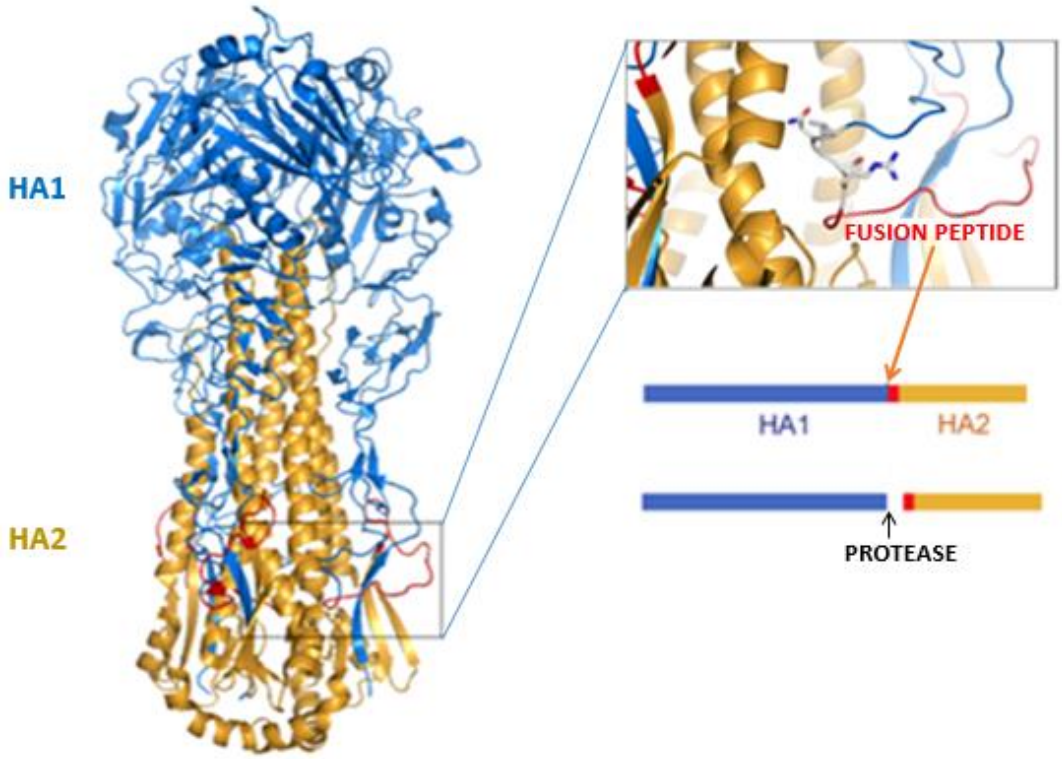


Figure 3. Cleavage of the influenza HA protein. Specific cellular proteases are responsible for cleaving the HA between HA1 and HA2 subunits and exposing the fusion peptide (modified from Hamilton et al., 2012).

Viral RNPs are trafficked into the host cell nucleus by nucleoprotein, where transcription and replication of the negative sense viral genome takes place. Transcription involves the production of capped, polyadenylated messenger RNA (mRNA) and positive strand complementary RNA (cRNA). Further transcription, using the cRNA as a template, produces complementary negative sense vRNA that is packaged into progeny virions. Post transcription capped and polyadenylated viral mRNAs are processed alongside host-cell mRNAs and, without the help of viral proteins, pass out of the nucleus to be translated. However, full-length vRNA is dependent on M1 and NEP proteins to exit the nucleus via nuclear pores (Cros & Palese, 2003).

Translation of viral proteins occurs on membrane-bound ribosomes on the rough endoplasmic reticulum. Post translation modification then takes place within the Golgi and signals for the surface glycoproteins HA, NA and M2 to assemble at the host cell surface. Original theories described a random vRNA packaging process whereby only virions containing complete genomes became infectious (Bancroft & Parslow, 2002). Subsequently however, a selective packaging process of all eight vRNA segments within the progeny virions prior to budding has been proposed (Fujii *et al.*, 2003). Once the progeny virions bud from the host cell the final stage of virus release depends on NA to cleave the sialic acid receptor bound to the HA on the surface.

1.2 Mechanisms of influenza virus evolution

The RNA polymerase enzyme, incorporated within the influenza viral genome, is relatively low fidelity and lacks the ability to proof read RNA transcripts (Szewczyk *et al.*, 2014). Consequently, an accumulation of random mutations within the genome occurs over time. Of the mutants that are produced, selection pressure allows those with a growth advantage over the parental strain to proliferate (Zambon, 1999). Changes to the HA surface glycoprotein, the major antigen on an influenza virion, are of importance as these changes can alter antigenic epitopes. Small changes to the surface glycoproteins may allow for immune escape in some individuals, which can result in an outbreak. The steady incremental change that leads to antibodies no longer recognising the HA is described as antigenic drift. Every year the World Health Organisation (WHO) meets to discuss necessary changes to human influenza vaccines in an attempt to protect against circulating strains. Therefore, identifying the mutations responsible for immune escape and vaccine failure is important. The rate of amino acid changes in human HA over a period of 14 years was reported as 0.87% (Skehel *et al.*, 1983), compared to 0.36% changes over 16 years for equine HA (Daniels *et al.*, 1985), these findings were confirmed in 1991 (Bean *et al.*, 1992).

The segmented nature of the RNA virus genome facilitates the possible exchange of genes with another influenza strain during co-infection and replication within a cell. If this reassortment event occurs between different virus subtypes and causes a change in one or both of the major surface glycoproteins, HA and/or NA, the change is referred

to as antigenic shift as these are major immune targets. Antigenic shift is often responsible for causing human pandemics. A potent reminder of such an event was demonstrated by the swine flu pandemic that spread to humans in 2009 (Isaacs, 2010). The notion of a pig acting as a 'mixing vessel' for influenza viruses had been suggested prior to the outbreak (Ito *et al.*, 1998; Yasuda *et al.*, 1991); two avian viruses and a human virus reassorted within the pig, acquiring the necessary genes to infect humans (Smith *et al.*, 2009). Such a dramatic change in the virus genome renders most, if not all, of a population susceptible to infection as no antibodies are present to combat infection. It is the continued transmission of influenza viruses to new host species that can potentially act as intermediate hosts, leading to human susceptibility, which causes particular concern.

1.3 Transmission of influenza virus

The dynamics of influenza virus transmission are complicated and it is difficult to predict how and when a pandemic may arise. There are two main factors to consider; the ecology of species and host range. Migration of birds (Dugan, 2012), changes to habitats (Sehgal, 2010), domestication of animal species (Munoz *et al.*, 2016) and conditions in food markets (Yu *et al.*, 2014), are all environmental factors that can affect transmission of influenza viruses. Wild aquatic birds are known to be the reservoir of influenza A viruses that have been transmitted to sea-mammals and land-mammals over time (Figure 4). The factors that determine host range are vast and not yet fully understood (Barclay *et al.*, 2014). Upon inhalation or consumption of the virus, the binding specificity of HA to sialic acid receptors can determine infection success. Different species contain different types and quantities of sialic acid receptors: the long-standing theory is that avian species contain more α-2,3 linked sialic acid receptors whereas humans contain more α-2,6 (Matrosovich *et al.*, 1997). Intermediate species such as swine have been highlighted as mixing vessels because they contain both α-2,3 and α-2,6 sialic acid receptors, which makes them susceptible to multiple infections (Imai & Kawaoka, 2012). Equids have more α-2,3 linked sialic acid receptors (Suzuki *et al.*, 2000) whereas the composition of receptors found in dogs and seals is often debated (Anthony *et al.*, 2012; Muranaka *et al.*, 2011). Furthermore, sialic acid receptors can be modified,

and due to their role in maintaining multiple cell functions, this may impact the host tropism of influenza viruses. For example, acetylation of the most common form of sialic acid receptor N-acetylneuraminic acid (Neu5Ac) at carbon atoms 4, 7, 8 or 9 can occur and such chemical changes may interfere with host-pathogen interactions. Recently developed probes have been used to identify modified sialic acid receptors in influenza host species/animal models, specifically those that are acetylated in positions 4, 9 and 7,9. Results indicated that 9 and 7,9-O-acetyl modified receptors were much more abundant in a variety of hosts including humans, pigs, guinea pigs horses, dogs and ducks whereas 4-O-acetyl receptors were only found in guinea pigs and horses (Wasik *et al.*, 2017). As well as chemical additions to the sialic acid receptors, a single amino acid mutation within the receptor binding site of the HA protein can alter the preferential binding to a different form of sialic acid receptor (Connor *et al.*, 1994). It is also noteworthy that although the HA protein is known to play an important role in the transmission of the influenza virus, other proteins have also been shown to impact the process (reviewed in Barclay et al., 2014). Surveillance of circulating influenza viruses is a necessity in order to try and identify the on-going changes within virus genomes and shifts in host range.

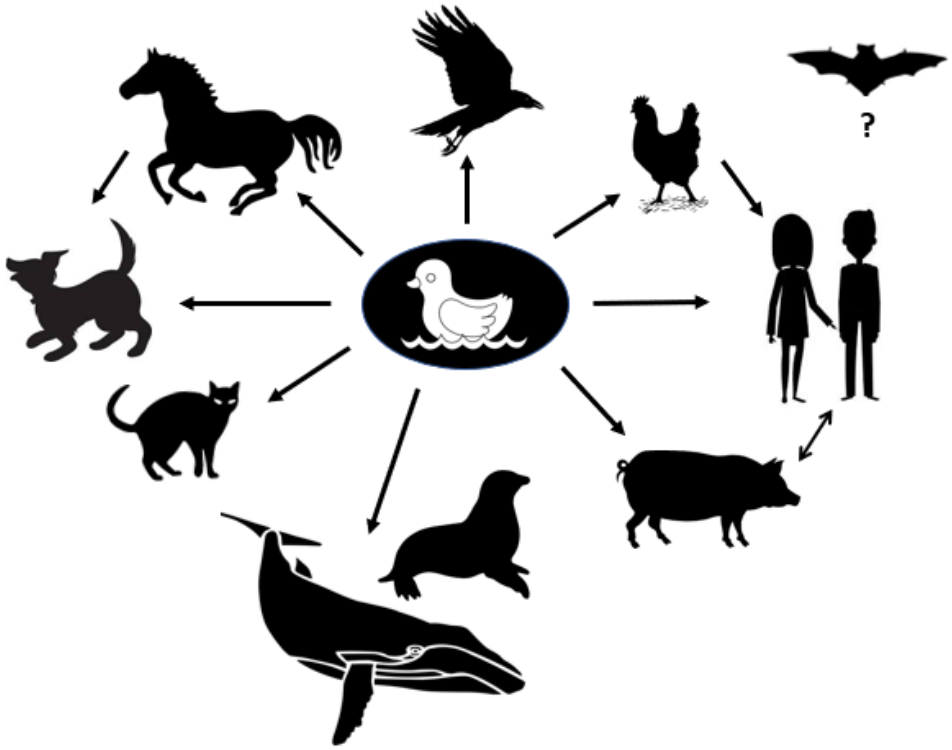


Figure 4. Transmission of influenza virus between different host species (modified from Manz *et al.*, 2013).

1.4 Neglected influenza hosts

Aside from the human, avian and swine viruses that form the main focus of influenza research, other species remain relatively neglected. Horses, dogs and seals are examples of such 'neglected' species that have all been detrimentally affected by outbreaks of influenza.

1.4.1 Equine influenza

Equine influenza (EI) causes disease amongst equids worldwide. Naïve horses infected with EI typically present with the following clinical signs; coughing, nasal discharge, pyrexia and lethargy. Mortality rates as high as 20% from primary influenza A type virus infections have been reported (Guo *et al.*, 1991; Yondon *et al.*, 2013). However, fatalities are more likely in young horses that suffer from secondary bacterial infections (Patterson-Kane *et al.*, 2008; Peek *et al.*, 2004). The close proximity of stable-kept horses facilitates rapid transmission of EI. Furthermore, EI is highly infectious, with large quantities of virus expelled during coughing episodes. The global movement of horses heavily influences transmission of EI. The equine sporting industry, including horse racing and polo, involves transporting horses around the world to participate in competitions. Outbreaks of the disease have affected race meetings since the late 1970s and can lead to the cancellation of events: Clinical signs of the disease appear more severe when a horse continues to exercise (Gross *et al.*, 1998). As well as participation in sport, equids remain a very valuable working animal in developing countries and as such, outbreaks of EI in countries such as India are a concern (Virmani *et al.*, 2010). Prior to introducing horses to a new population it is essential to place the horses in quarantine in order to prevent an outbreak from occurring (Morley *et al.*, 2000). A potential complication during the quarantine procedure is that vaccinated horses may have a subclinical infection and therefore be released with the potential to transmit the virus. There are very few nations that have not experienced EI (Cullinane & Newton, 2013). Australia was considered EI-free until 2007 when a quarantine procedure failed, leading to an outbreak that affected thousands of horses (Webster, 2011). The Australian government spent in excess of $1 billion to control the disease (Callinan, 2008). These circumstances highlight the importance of controlling EI.

1.4.1.1 Immune response

The non-specific, innate immune response to influenza virus infection aims to contain and prevent propagation of the virus. The innate response is thought to be initially triggered by cellular pattern recognition receptors, which induce an antiviral state within infected and surrounding cells, usually via the interferon pathway (Rehwinkel *et al.*, 2010). Other non-specific immune mechanisms that aim to limit viral spread include lymphocytes, complement and the activation of macrophages. This first line of defence is followed by a specific immune response.

In order to combat an influenza infection, cell-mediated and humoral immunity are both essential (Hannant *et al.*, 1989; Hannant & Mumford, 1989). Cell-mediated responses can be considered as heterologous due to the broad range of peptides that T-cells can recognise, some of which are conserved among different strains and subtypes of influenza. The importance of cell-mediated responses to EI infections was made apparent by a study that demonstrated how ponies with very low levels of antibodies against HA, as measured by SRH, retained clinical and virological protection one year post infection with EI virus (Hannant *et al.*, 1988). Consequently, it is desirable for vaccines to induce cytotoxic T lymphocyte responses (CTL) as well as specific antibody responses (Slater & Hannant, 2000). Assays for equine cell mediated responses against influenza have been developed and implemented (Adams *et al.*, 2011; Paillot *et al.*, 2007). However, it is important to note that cell mediated responses do not provide protection against infection. T-cells function to eliminate a viral infection and thus cannot be used to determine if an individual will be protected. Antibody levels, derived from natural infection or vaccination, can be measured to ascertain protective thresholds.

When challenged with an influenza virus, neutralising antibodies provide subtype specific protection by preventing viral entry into host cells. The antibody response is primarily driven against the surface glycoprotein HA. Horses have 11 immunoglobulin isotypes IgG (7 subclasses), IgA, IgM, IgE and IgD; each involved in aspects of the immune response against different pathogens. Antibody isotypes IgA (mucosal) IgGa and IgGb (serum) are associated with natural protection against influenza infection (Nelson *et al.*, 1998). Monoclonal antibodies are manufactured and

used to investigate the equine immune response, such as quantifying isotype concentrations in the horse (Keggan *et al.*, 2013). Our knowledge and further understanding of the equine immune response is important if we are to produce effective vaccines. Different approaches to producing effective vaccines are continuously being reviewed (Paillot *et al.*, 2007). Interestingly, similarities between human and equine immune responses to influenza have recently been highlighted, which supports the potential use of horses as an animal model for influenza infection and emphasises the importance of studying the equine immune response (Horohov, 2015).

1.4.1.2 Diagnostics

Diagnosis of an EI infection is typically achieved by direct detection of viral antigens or genetic material contained in nasal swab samples. There are many different diagnostic tests available however the sensitivity and specificity varies greatly between them (Yamanaka *et al.*, 2016). The current gold-standard is RT-PCR (Quinlivan *et al.*, 2005). Alternatively, indirect detection of the virus can be attained through serological assays (Zimmerman & Crisman, 2008). These assays identify the presence of antibodies against EI within test blood sera. If the individual is seronegative in the acute phase (blood sample taken upon the onset of clinical signs) and seropositive in the convalescent period (sample taken around two weeks later) the individual is said to have seroconverted, which is indicative of infection.

1.4.1.3 Surveillance

Surveillance is an essential part of the process to produce a vaccine that will provide protection against circulating EI viruses. Reports of 'influenza-like' illness date back to the 1200s and were particularly prevalent in the United States during the 1800s (Morens & Taubenberger, 2010). Two subtypes have been established within the equine population; H7N7 and H3N8. The first confirmed report of an EI infection was H7N7 in 1956, Prague, Czechoslovakia (Sovinova *et al.*, 1958). This virus subtype has not been

isolated for more than 20 years and is therefore considered extinct (Webster, 1993). The H3N8 subtype was first identified in Miami, Florida, 1963 (Waddell *et al.*, 1963) and continues to circulate globally. In the late 1980s, initially based on the geographical distribution of viruses, H3N8 diverged into Eurasian and American-like lineages. Notably, American-like viruses were also isolated in Europe and vice versa (Daly *et al.*, 1996). Furthermore, in 2001, it was reported that the American lineage had further diverged into three sub-lineages; South American, Kentucky and Florida (Lai *et al.*, 2001). At present, the Florida sub-lineage is divided into two clades; 1 & 2. Both Florida clade 1 and 2 viruses continue to circulate in Europe while clade 1 predominates in America (Bryant *et al.*, 2011).

1.4.1.4 Vaccination

Vaccines against EI were made available in the late 1960s and became mandatory for thoroughbred racehorses under the Jockey Club Rules of Racing in March 1981. Other regulatory equine bodies including the British Show Jumping Association (BSJA) and FEI (Federation Equestrian International) also impose a mandatory vaccination programme for horses participating in national and international competitions, respectively. Mandatory vaccination programmes aid the control of the disease, however, not all horses fall within the categories for mandatory vaccination. For the 'everyday' working or non-competition horse, vaccination is recommended to their owners but they are not obliged to comply. Herein, there are two major hurdles to consider; the control of EI transmission via unvaccinated populations and in the instances where vaccination is employed, to ensure that the component EI vaccine strains are up to date and provide optimal protection.

Although EI vaccines were in use by the late 1960s, major UK outbreaks in 1979 and 1989 highlighted the need for vaccine strains to be updated. Moreover, due to the phylogenetic diversity of EI viruses, vaccine strains representative of the different lineages were suggested for inclusion (Daly *et al.*, 2004; Woodward *et al.*, 2015; Yamanaka *et al.*, 2015). Pony challenge studies are conducted to test the efficacy of vaccine strain components, however, it would be preferable to avoid these studies and

employ a sequence-based approach (Daly & Elton, 2013). The difficulty is that vaccine updates cannot simply be determined by the number of amino acid changes that appear in the HA, as a result of antigenic drift. An understanding of the key amino acid changes that infer antigenic differences are crucial to optimising protection (Woodward *et al.*, 2015). In the last decade, outbreaks amongst vaccinated populations have continued to occur due to the use of out-dated vaccines (Newton *et al.*, 2006; Yamanaka *et al.*, 2008). At present in the UK, there is only one EI vaccine that meets the recommendations of the OIE (World Organisation for Animal Health). The development of new effective vaccines is continuously underway but implementing production and marketing is a slow process (Paillot *et al.*, 2006, 2016).

1.4.2 Canine influenza

Canine influenza (CI) disease causes similar clinical signs to that of equine influenza; coughing, nasal discharge, fever and lethargy. Furthermore, cases of mortality due to haemorrhagic pneumonia have been recorded (Yoon *et al.*, 2005).

1.4.2.1 Transmission and surveillance

Influenza A subtypes H3N8 and H3N2 emerged in the canine host via separate transmission events from equine and avian origins, respectively. The history of CI is relatively short-lived as the first outbreak was recorded only 13 years ago. In 2004, the H3N8 subtype was identified in the canine host at a Greyhound racing track in Florida, USA (Crawford, 2005). The dogs at the facility had shown signs of respiratory disease, prior to the official isolation of influenza virus, but at the time the cause of disease was unknown. Serological assays on archived blood samples did however indicate that the virus was circulating in the USA as early as 1999 (Anderson *et al.*, 2012a). In 2005, different breeds of dog were also noted to be affected by the disease (Payungporn *et al.*, 2008). Genetic analysis confirmed that there was a direct transmission of the H3N8 virus from horse to dog, in the US, with more than 96% nucleotide similarity to the original equine strain (Crawford, 2005). Investigations into how the virus was directly

transmitted found two possible explanations; inhalation via close contact (Yamanaka *et al.*, 2009) and the consumption of infected horse meat (Daly *et al.*, 2008). The independent transmission event of the H3N8 virus in the United Kingdom was reported after the outbreaks in the US (Daly *et al.*, 2008) through re-examination of formalin-fixed paraffin-embedded tissue from a Foxhound that suffered with similar clinical signs. A partial HA sequence was recovered from the tissue and found to be related to recent EI virus strains (Janet Daly pers. comm.).

In 2006, the first case of a canine H3N2 virus was reported, in a pet dog in Guangdong, China (Li *et al.*, 2010). Within five years it was isolated from roaming dogs in China (Su *et al.*, 2013) and furthermore, the virus was isolated in South Korea in 2007 (Song *et al.*, 2008). Similarly to the US H3N8 transmission event, direct transmission and/or the consumption of infected meat were thought to be the cause (Song *et al.*, 2008). Although H3N8 predominates in the US and H3N2 in Asia, recent studies have shown that the H3N2 virus is circulating in the US (Centers for Disease Control and Prevention, 2015) and there is serological evidence to suggest that H3N8 is present in China (Zhou *et al.*, 2016).

One of the initial, intriguing characteristics of CI was the ability of the virus to transmit between dogs in a relatively short amount of time. Most novel transmission events involve a single round of infection and do not establish within a new population. Occasionally viruses are transferred, spread efficiently and become established in a new population, as was demonstrated by CI. The genetic diversity of the virus within the canine host was studied and eluded to the transient nature of mutations that may enable antigenic escape or an increase in host range (Hoelzer *et al.*, 2010). The transmission of canine influenza viruses, not only to other animals but to humans, is a particular concern as dogs are domestic companion animals. American Pet Products Association estimates that there are 89.7 million pet dogs in the US, based on the 2016 National Pet Owners Survey and The National Bureau of Statistics of China reports that there are 27.4 million pet dogs. A novel canine H3N1 virus has evolved via an antigenic shift event in which the pandemic H1N1 2009 and canine H3N2 strains reassorted (Song *et al.*, 2012). This novel virus, identified in Korea, highlights the permissibility of the canine host to human influenza infections.

1.4.2.2 Vaccination

In 2009, the first canine influenza inactivated vaccine against the H3N8 subtype was shown to significantly reduce the severity of clinical signs seen in puppies, as well as the quantity and duration of viral shedding (Deshpande *et al.*, 2009). Nobivac® Canine Flu H3N8 was produced by Merck Animal Health and is licensed for use in the US. The inactivated H3N8 vaccine also provides some protection against the H3N2 subtype; Antibodies are elicited against the more conserved stalk region of the HA and internal viral proteins but do not eliminate infection (Willis *et al.*, 2016). Furthermore, two live-attenuated vaccines against H3N8 have been developed using different strategies; modification of the NS1 protein (Nogales *et al.*, 2017a) and a temperature sensitive mutant virus (Nogales *et al.*, 2017b). Both live-attenuated vaccines induced protection against the homologous H3N8 canine influenza virus in mice and discussions as to which vaccine is the best candidate for canine trials are underway.

In 2013, a virus-like particle (VLP) vaccine was designed specifically to target the H3N2 subtype. The vaccine includes the H3 and M1 proteins and prevents any clinical signs of disease, reduces pulmonary pathology and viral shedding (Lee *et al.*, 2013). In China, inactivated H3N2 vaccines have also been developed that prevent disease and reduce viral shedding (Cureton *et al.*, 2016; Lee *et al.*, 2010). Although the vaccines do not eliminate viral shedding, the reduction should help to reduce transmission of the virus at present. In addition, a live-attenuated H3N2 vaccine has recently been developed and induced a higher protection efficacy, during a challenge study in mice, than the inactivated H3N2 vaccine (Rodriguez *et al.*, 2017a).

It is noteworthy that the live-attenuated monovalent vaccines do not provide cross-protection against the heterologous virus strain. Therefore, the most promising candidate to date is a live-attenuated bivalent vaccine that does provide protection against both H3N8 and H3N2 subtypes (Rodriguez *et al.*, 2017b).

1.4.3 Phocine influenza

Phocine (seal) influenza A virus outbreaks have been reported since the 1970s in different species (Fereidouni *et al.*, 2014). In 1979 along the New England Coast, US, an epizootic involving an H7N7 subtype (A/seal/ Massachusetts/1/80) killed more than 400 harbour seals (*Phoca vitulina*) (Geraci *et al.*, 1982). Periodic outbreaks with other subtypes have also occurred in the US; H4N5 in 1982 (Hinshaw *et al.*, 1984), H4N6 in 1991, H3N3 in 1992 (Callan *et al.*, 1995) and H3N8 in 2011 (Anthony *et al.*, 2012). Furthermore, the H10N7 subtype was isolated from seals in Sweden, Denmark, Germany and the Netherlands (Bodewes *et al.*, 2015; Zohari *et al.*, 2014). Interestingly not all seal species appear to be clinically affected by the disease: Grey seals (*Halichoerus grypus*) do not present with clinical signs and it has recently been proposed that the seal may act as a form of reservoir for the disease (Puryear *et al.*, 2016). Hooded seals (*Cystophora cristata*) and harp seals (*Pagophilus groenlandicus*) have also been identified as seropositive for influenza A infection but clinical signs were not recorded during sampling and so whether or not these animals were affected by the disease is unknown (Stuen *et al.*, 1994).

For the purpose of this thesis the H3N8 subtype is of particular interest because it also infects equine and canine species. It is interesting that H3N8 is found in a variety of species and is frequently isolated from aquatic birds (Hill *et al.*, 2012). The emergence of the H3N8 subtype killed 162 harbour seals in New England, US (Anthony *et al.*, 2012). Phylogenetic sequence analysis indicated that the subtype was of avian origin. The receptor binding specificity of the seal H3N8 virus was found to have an increased affinity for α2,6 linked sialic acid receptors, which are associated with human influenza virus infections, compared to α2,3. The virus infected human lung cell cultures and could undergo respiratory transmission in a ferret model (Karlsson *et al.*, 2014). Although seals are less likely to come into contact with humans than dogs and horses, the virus still poses a potential threat to human health. Haemagglutinin Inhibition (HI) assays were performed using the A/harbour seal/Massachusetts/1/2011 (H3N8) virus against pre and post vaccination human H3 seasonal vaccine serum samples, resulting in no evidence of cross-reactivity. Therefore, this suggests that the seal H3 virus is significantly

different to the human vaccine strains and thus the human population could be susceptible to the virus if interspecies transmission occurred (Karlsson *et al.*, 2014).

The transmission of human influenza viruses to seals has been documented: The human pandemic H1N1 2009 virus was isolated from Northern Elephant Seals (*Mirounga angustirostris*) on the central California coast. There was speculation that the virus may have been transmitted via aquatic birds at sea, however phylogenetic analysis confirmed high levels of similarity and an ancestral link that suggested a direct transmission from humans was more likely. As mentioned previously, it is unlikely that humans and seals have direct contact at sea, however water infected with human excrement could be a source of infection (Goldstein *et al.*, 2013). In Russia, antibodies specific to a human H3N2 virus were detected in Baikal Seals (*Phoca sibirica*) and Ringed Seals (*Phoca hispida*) (Ohishi *et al.*, 2004). These incidences further highlight the importance of monitoring influenza viruses in seals due to the potential transmission between species.

1.5 Serological Assays

Further to using serological assays in diagnosing influenza infections, they are of great importance for global surveillance of the disease, subtyping isolates and in vaccine efficacy testing procedures (OIE World Organisation for Animal Health, 2016). Current assays in clinical use include the haemagglutination inhibition assay (HI), single radial haemolysis (SRH), enzyme-linked immunosorbent assay (ELISA), and virus neutralisation assay (VN). All serological assays detect the presence of antibodies within test blood sera. However, the application and interpretation of each assay is different.

1.5.1 Haemagglutination inhibition assay (HI)

The HI assay, developed in the 1940s, detects decreases in HA-binding to erythrocytes when antibodies are present within serum (Hirst, 1942). Serum must be pre-treated, with potassium periodate, kaolin or receptor-destroying enzyme, and heat inactivated to remove any non-specific agglutinins. Pre-treatment of serum is crucial to avoid false positive results. The consequences of ineffective pre-treatment were demonstrated when using kaolin to test for antibodies against equine H7N7 (Boliar *et al.*, 2006). A Tween 80/ether solution can be added to the antigen, to prevent viral infectivity and cross-contamination, whilst increasing HA activity for H3N8 subtypes. However, a consequence of increasing sensitivity is a reduction in specificity (Mumford, 1992). The assay read-out is an HI titre; the reciprocal of the serum dilution at which agglutination is completely inhibited. The requirement for classification of seroconversion, between acute (onset of clinical signs) and convalescent (two weeks later) serum samples, is a minimum four-fold increase in titre (Morley *et al.*, 1995). The OIE provide a detailed protocol for the HI assay (OIE, 2016). Figure 5 illustrates A) expected observations and B) an example assay. In practice, results are not always straightforward to interpret and well-trained personnel are a necessity to accurately read an HI plate.

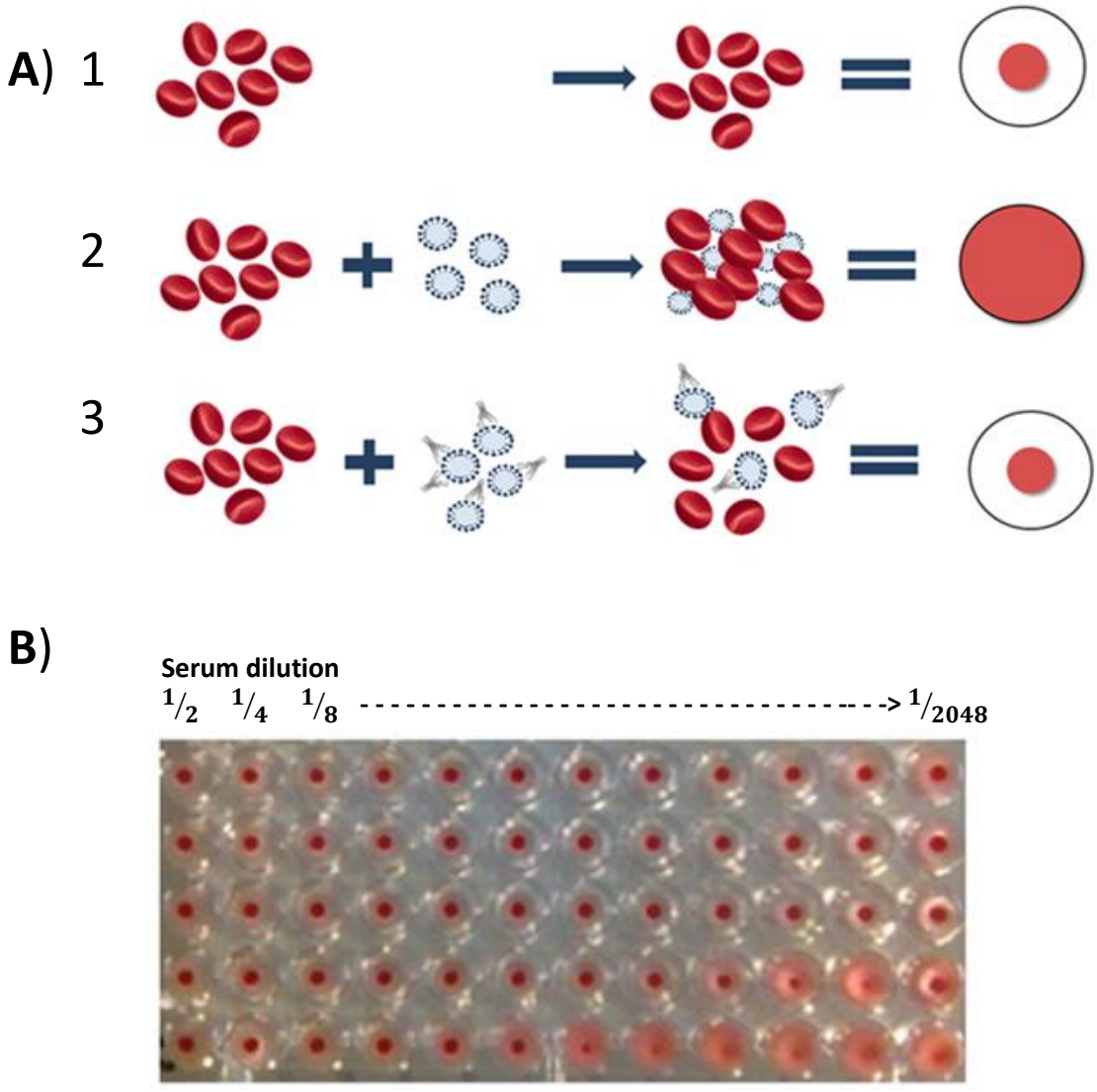


Figure 5. A) Principles of the haemagglutination inhibition assay. 1 – Red blood cells (RBCs) alone do not agglutinate and thus a red pellet of cells forms at the bottom of a V-shaped well. 2 – Virus added to RBCs, the virus binds to sialic acid receptors on the RBCs causing cells to agglutinate. A uniform shade of red is observed due to the formation of a cell–virus lattice. 3 – Upon addition of an antibody directed against the HA, the influenza virus is prevented from binding to the RBC thus agglutination is inhibited and a red pellet of cells is observed. **B)** Example HI assay plate.

1.5.1.1 HI applications: Advantages and disadvantages

HI assays have multiple applications including HA subtyping, surveillance and vaccine testing. Recently HI was used to evaluate the seroprevalence of equine influenza in Israel (Aharonson-Raz *et al.*, 2014) and Nigeria (Olusa & Adeyefa, 2009) as well as seal influenza in the Netherlands (Bodewes *et al.*, 2015) and canine influenza in China (Zhou *et al.*, 2016). Furthermore, HI is the global standard for antigenic characterisation of new isolates. Characterisation is achieved by a comparison of HI titre to a panel of reference sera. Isolates from Europe, Dubai and the USA were recently characterised in an attempt to improve surveillance of equine influenza (Woodward *et al.*, 2014). In order to effectively discriminate between isolates, strain-specific antibodies are beneficial. Ferret anti-serum is typically more strain-specific than equine serum and is therefore the preferred source (Burrows & Denyer, 1982). Furthermore, data from HI assays using ferret anti-serum, are used to aid decision making on component equine influenza vaccine strains (Bryant *et al.*, 2011).

HI is officially recommended by the OIE for vaccine testing. It is also accredited by the Committee for Proprietary Medicinal Products (CPMP) as a determining factor for human vaccine licensing. In humans, a post vaccination HI titre of >1:40 is considered to be the protective threshold value, reducing the chance of natural infection by 50%. Recently, the US Food and Drug Administration (FDA) applied this threshold for licensing pandemic human influenza vaccines. Recent reviews on serological techniques used for human vaccine evaluation highlight the important issue of defining 'protection' (Cox, 2013; Trombetta *et al.*, 2014). For equine influenza, a mean HI titre of ≥1:64 is required to induce clinical protection. However, a higher titre may be necessary to confer protection against viral shedding (European Medicines Agency, 2014).

A drawback to using the HI assay is the type of antibody that it quantifies. The HI assay focusses on HA binding to sialic acid residues on red blood cells, therefore measuring antibodies that bind to the trimeric head of HA and prevent haemagglutination. Neutralising antibodies correlate with protection and do not only bind to the head of HA, therefore HI titres are not an exact measure for protection. That considered, one report has shown good correlation between the HI and a virus neutralisation assay for equine (Morley *et al.*, 1995).

Reproducibility of an assay is important for the integrity of studies, particularly those used in vaccine efficacy testing. The HI assay is simple to perform but the red blood cell component is difficult to standardise. The inter-laboratory variability of the assay is a well-documented issue. A four-fold difference in HI titres was documented between different laboratories for equine influenza (Daly *et al.*, 2007a). Furthermore, in human influenza studies, greater than 80% variation has been recorded (Wood et al., 1994, 2012). Despite the issues, HI remains a conventional assay for equine, canine and phocine influenza due to its simplicity and minimal associated costs. It is particularly favourable for diagnosis and surveillance in resource-poor countries.

1.5.2 Single radial haemolysis (SRH)

SRH has been used since the late 1970s to detect antibodies against influenza (Schild *et al.*, 1975). The assay identifies antibodies in blood serum by utilising the cell lysis properties of complement. Serum is added to an agar plate containing virus-coated RBCs and, if anti-HA antibodies are present, the complement factors bind to antibody/antigen (HA) complexes on the influenza virus covered RBCs creating a zone of haemolysis (Figure 6). The level of strain-specific antibodies correlates with the size of the zone of lysis. Furthermore, seroconversion between acute and convalescent serum samples is defined by a 25mm^2 or a 50% increase (whichever is smaller).

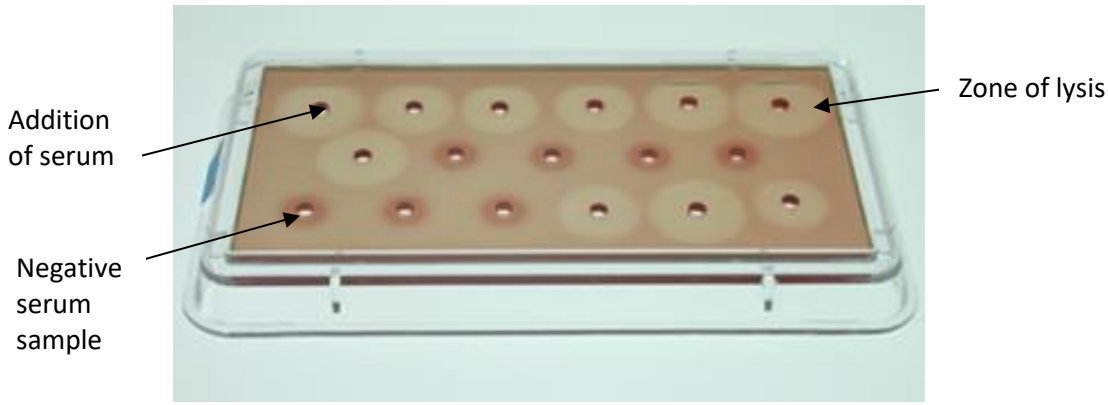


Figure 6. Example of an SRH assay plate. Serum is added to the wells within the agar and if anti-HA antibodies are present a zone of lysis forms (highlighted in the image).

1.5.2.1 SRH applications: Advantages and disadvantages

The OIE considers SRH to be of equal merit to the HI assay for equine influenza vaccine testing (OIE, 2008). There is a strong correlation between post-vaccination protective immunity and antibody detection in the SRH assay, hence why it is a favourable choice for testing vaccine immunogenicity. Another benefit of using SRH is the defined correlates of protection; antibody levels >150mm^2 infer virological protection against homologous vaccine-challenge strains, clinical protection is seen between 85 and 150mm^2 and between 50 and 85mm^2 only partial protection is seen (Mumford & Wood, 1991). These measurements are used to determine when vaccine strain updates are necessary (Gildea *et al.*, 2013).

The serum is not diluted in an SRH assay, which provides a linear readout compared to the titres used in HI. This makes SRH more sensitive than HI, hence a 50% increase in the zone of lysis will confirm seroconversion compared to a four-fold increase for HI. The assay is more reproducible than HI (Mumford & Wood, 1991, 1993). However, it is not without variation; up to 3.9-fold differences were calculated across nine laboratories for equine influenza assays (Daly *et al.*, 2007a). SRH is not favourable for diagnostic purposes because the assay does not detect IgM antibodies seen in the early stages of infection.

1.5.3 ELISA

An ELISA can be used to detect antibodies that bind viral proteins; typically the structural nucleoprotein (NP) is targeted (Ji *et al.*, 2011). The ELISA is a flexible assay platform with variations such as; blocking, indirect, competition and cell-based.

1.5.3.1 ELISA applications: Advantages and disadvantages

An ELISA is quick to perform and high-throughput, which is beneficial for diagnostic purposes. Similar to HI, the ELISA is not necessarily representative of a neutralising antibody response due to the semi-quantitative measure of antibody binding. Furthermore, anti-NP antibodies are not subtype-specific and therefore other tests are

necessary to identify particular strains. For example, serosurveillance of seal influenza was conducted using both ELISA and HI assays (Bodewes *et al.*, 2015). During the equine influenza outbreak in Australia 2007, the ELISA was the chosen assay for screening purposes, however some results were inconclusive and a follow-up HI test was actioned (Sergeant *et al.*, 2011). A very useful aspect of the ELISA is the ability to Differentiate Infected from Vaccinated Animals (DIVA) in some cases. For example, animals vaccinated with subunit vaccines will not raise antibodies to all viral proteins whereas individuals who have been infected with a virus will. Therefore, depending on the protein measured, the ELISA can give differential results (Galvin *et al.*, 2013; Kirkland & Delbridge, 2011). A cell-based ELISA measuring the non-structural NS1 protein has been suggested for DIVA as theoretically, only infected equines should mount an immune response to the non-structural NS1 protein, not those vaccinated with inactivated virus (Wojciech *et al.*, 2011).

1.5.4 Virus neutralisation (VN)

Virus neutralisation assays identify antibodies that are capable of inhibiting virus entry and or preventing replication within cells (Han & Marasco, 2011). Different techniques have been developed, but all VN assays measure biological function, specifically, a reduction in virus particle infection. The 'gold standard' is the plaque reduction neutralisation test (PRNT); serum is incubated with a standard amount of antigen and added to a monolayer of permissive cells, then an overlay of agarose or carboxymethylcellulose is necessary to prevent the virus spreading within the supernatant. Plaques formed are then analysed by microscopy or eye often following cell staining. Micro-neutralisation (MN) is a cell-based assay that incorporates a standard amount of antigen and a serial dilution of serum in a 96-well plate. The neutralising ability of antibodies can be measured by the presence/absence of cytopathic effect (CPE) or the quantity of virus within the supernatant, which is usually measured by an ELISA. Alternatively, serum-virus mixtures can be added to the allantoic cavity of embryonated hens' eggs, harvested and used in a haemagglutination assay to determine serum titres (Yamanaka *et al.*, 2015).

1.5.4.1 VN applications: Advantages and disadvantages

As well as measuring biological function, VN assays are sensitive, and are therefore well-suited for measuring vaccine-induced responses. Furthermore, the results can be used to advise on necessary vaccine strain updates (Ozaki *et al.*, 2001; Yamanaka *et al.*, 2015). VN assays would also be favourable for diagnosing infection but the associated costs are high compared to HI. The assays are not typically high-throughput, however fixing cells on a MN plate and using an NP ELISA to quantify virus can be achieved in 24 hours (Khurelbaatar *et al.*, 2014). It is also important to note that infectious virus is necessary for VN assays and thus appropriate bio-containment protocols must be followed. Another limiting factor of VN is the limited CPE of some influenza viruses (equine) on commonly used cell lines, for example MDCK cells (Madin-Darby canine kidney). The assay has, however, been successfully implemented for canine influenza serology (Crawford, 2005) despite the difficulty in implementation for routine testing (Dubovi, 2010).

1.5.5 HA Microarray

The HA protein microarray has been developed as a high throughput assay that detects antibody responses against a range of different HA subtypes (Koopmans *et al.*, 2012). Antigen protein is spotted, usually in triplicate, on to a nitrocellulose-coated glass slide and the slides are left in a dark, drying chamber until use. A serial dilution of serum is added to the slide for one hour at 37^{o}C in a moist chamber, then washed with a protein array wash-buffer before adding a secondary antibody conjugated with a fluorescent dye. After washing again with the wash-buffer and water, the signal can be quantified using appropriate fluorescence detection equipment.

1.5.5.1 HA Microarray applications: Advantages and disadvantages

The microarray was designed as an alternative to cell-based assays, such as HI, to exclude the issue of red blood cell variability. The quantity of serum required is minimal at 10µl and the durability of the assay has proven promising in dry storage conditions at 21^{o}C for up to 21 months (Freidl *et al.*, 2017).

1.6 Pseudotyped viruses (PVs)

PVs are chimeric virions that display a surface glycoprotein from a virus of interest, and typically contain a retroviral core (Temperton *et al.*, 2015). Gene deletions within the core prevent viral replication (particles can enter target cells but no progeny are produced) thus the virion is safer to work with. Elements of retroviral functionality are retained with structural and enzymatic genes (reverse transcriptase and integrase), which facilitate the self-assembly of virions and integration of a reporter gene carried by the remaining viral genome into a target cell genome of choice (Temperton *et al.*, 2007). The morphology and size of an influenza pseudotyped virus is very similar to that of an influenza A virion, approximately 100nm (Nefkens *et al.*, 2007), the length of the haemagglutinin spikes are also similar to an influenza A virion between 9-11nm (Sawoo *et al.*, 2014). The density of haemagglutinin on a lentiviral PV surface has not been characterised, however, reports suggest that it can vary; "At the protein level, all pseudotyped viruses have a composition similar to that of the wild-type virus, with the exception of the levels of HA, which appear to be slightly reduced" (Martinez-Sobrido et al., 2010) and "HA incorporation levels in each type of particle varied" (Hsu et al., 2015). Following a systematic analysis, a consensus protocol for influenza PV production has been published (Carnell *et al.*, 2015). Different reporter genes can be used during PV production, depending on the facilities available and project budget (Figure 7).

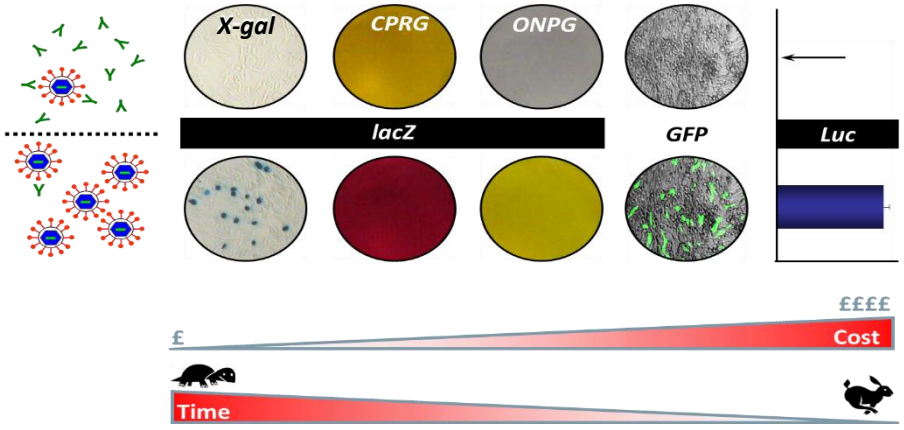


Figure 7. Examples of reporter genes used in pseudotype virus production with associated assay time and cost (modified from original that was kindly provided by Dr Edward Wright). The lower half of the figure illustrates the readout seen when PVs infect cells and the upper half of the figure illustrates the change in readout due to the presence of antibodies that prevent the PV from entering the cell and therefore the reporter gene is not expressed.

X-gal, CPRG (chlorophenol red-β-D-galactopyranoside) and ONPG (o-nitrophenyl-β-D-galactopyranoside) all involve colorimetric changes induced by β-galactosidase activity encoded by the lacZ gene. GFP (green fluorescent protein) indicates PV infectivity through fluorescence and upon cell lysis luciferase (Luc) catalyses the conversion of Luciferin into Oxyluciferin and light, the light is detected and recorded as relative luminescence units.

1.6.1 Pseudotype virus neutralisation assay (PVNA)

PVs are used in PVNAs to determine the presence of neutralising antibodies within serum samples. A knock-down in luminescence, reduction in fluorescence or a colour change, depending on the reporter system used (Figures 7 and 8), indicates the presence of neutralising antibodies against the envelope protein that inhibit PV entry into a target cell line of choice. An IC_{50} (the reciprocal of the serum dilution that causes 50% neutralisation of the PV) is typically reported after 48 hours.

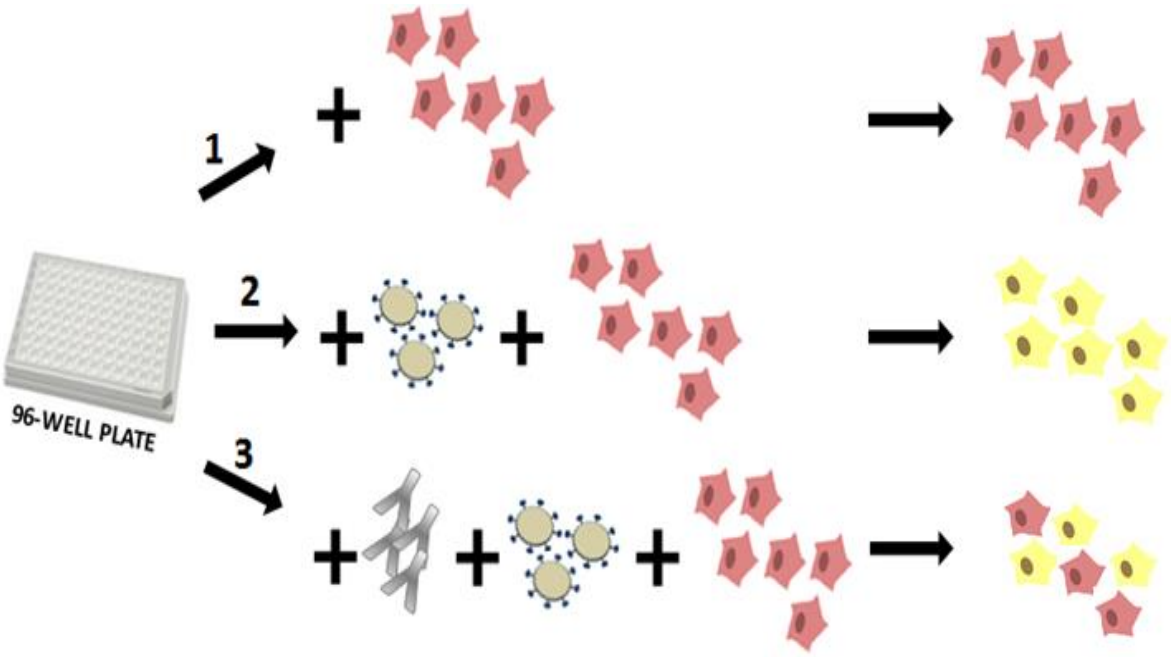


Figure 8. Principles of the pseudotype virus neutralisation assay using the luciferase reporter gene. **1 –** Target cells alone do not luminesce. **2 –** Virus added to target cells, the virus infects target cells and the reporter gene is integrated into the cell genome causing cells to luminesce. **3 –** Upon addition of antisera containing antibodies directed against the HA, the influenza pseudotyped virus is prevented from transducing target cells thus infection is reduced and the luminescence measured, decreases (Kinsley *et al.*, 2016).

1.6.2 PVNA applications: Advantages and disadvantages

The PVNA is a novel assay employing the safer, chimeric PV (as an alternative to infectious wild-type virus) to measure a neutralising antibody response. The PVNA provides a platform to research highly pathogenic viruses in a safer environment. A multiple plasmid transfection technique is used to produce the PVs, which facilitates an opportunity for individual gene manipulations that are potentially useful to study specific mutations. The costs associated with the PVNA can be higher than HI and thus it is less favourable in a diagnostic setting, however measuring the neutralising antibody response is beneficial for vaccine efficacy testing. The assay requires a minimal amount of serum, 2–10µl per replicate. Moreover, the neutralising antibody response to multiple viruses can be measured within a single assay by using two different reporter genes 'multiplexing', further reducing the quantity of serum used (Molesti *et al.*, 2014).

The PVNA was compared to traditional assays such as HI and MN for human influenza studies to assess whether PVs can act as substitutes for wild-type virus. The results demonstrated that the PVNA detects similar antibody responses with increased sensitivity (Garcia *et al.*, 2010; Kong *et al.*, 2006; Yang *et al.*, 2014). Emerging diseases pose a serious health and safety threat and thus the PVNA is being considered a 'next generation assay' for a potential solution to this problem (Mather *et al.*, 2013). Equine influenza PVs have been produced and trialled in PVNAs; the results showed 65% correlation with SRH results. There was an evident increase in sensitivity as some samples defined negative by SRH were deemed positive by the PVNA (Scott *et al.*, 2012).

1.7 Comparisons of assays

HI and SRH assays were established in the early 1930s and 1970s, respectively, and are still relied upon today. HI is inexpensive and quick to perform, lending itself for use in a diagnostic setting. Alternatively, SRH is more sensitive than HI and measures complement-mediated lysis, which is beneficial for vaccine immunogenicity testing. A disadvantage of employing either HI or SRH is the associated high level of inter-laboratory variability due to a lack of standardisation (Wood *et al.*, 2011). Vaccine immunogenicity testing requires a high level of consistency and accuracy and therefore the European Medicines Agency (EMA) suggest that a centralised laboratory should perform all assays for a vaccine study (European Medicines Agency, 2014). In such instances the VN assay is particularly beneficial, as the measurement of virus neutralising antibodies is most appropriate for evaluating vaccine immunogenicity. However, VN can be more variable than HI, is more expensive and difficult to reproduce (Stephenson *et al.*, 2007; Trombetta & Montomoli, 2016). Furthermore, particularly for equine influenza the VN assay can be complicated to perform due to the lack of CPE caused. The PVNA provides a potential solution to these problems but the reproducibility of the assay needs to be assessed, PV production optimised and the performance in studies for evaluating vaccine efficacy compared with standard assays.

2 MATERIALS AND METHODS

2.1 Molecular biology

2.1.1 Plasmids

The production of all lentiviral pseudotype viruses in this study was dependent on plasmid p8.91, which encodes the HIV gag-pol genes and facilitates viral particle formation. Due to the low copy nature of p8.91, the plasmid DNA was purified in bulk by GenScript®. Upon receipt of plasmid from GenScript®, the lyophilised pellets were resuspended in 20µl of double distilled (dd) H_2O (manufacturer guidelines). Firefly luciferase was the chosen reporter gene, encoded by pCSFLW, provided by Dr Temperton (Viral Pseudotype Unit, Kent).

HA and NA genes were sub-cloned into mammalian expression plasmid pI.18 using restriction enzyme (RE) sites present in the multiple cloning site (MCS) (Figure 9). Details of the restriction enzymes used for cloning individual genes are reported in the methods section within each chapter.

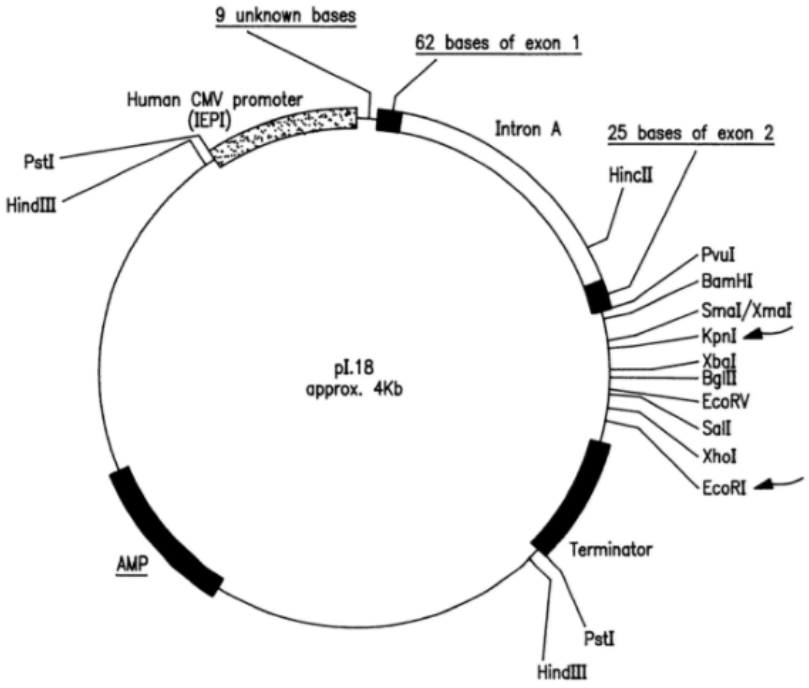


Figure 9. pI.18 mammalian expression plasmid with multiple cloning site. The plasmid was developed by I. Tarpey and N. Greenwood (US patent number US6187759 B1). The Human Cytomegalovirus (CMV) promotor facilitates expression of a gene cloned into the multiple cloning site and Intron A enhances expression. The plasmid harbours an ampicillin resistance gene (AMP) for clone selection.

2.1.2 Restriction enzyme digests (for cloning and screening purposes)

Fastdigest® restriction enzymes (RE) used were obtained from ThermoFisher Scientific. Table 2 indicates the quantity of DNA used per reaction along with other necessary reagents. Water and DNA volumes were adjusted depending on original DNA concentrations. Fastdigest® Green buffer was used as an alternative to adding loading dye when running digest reactions on an agarose gel (Methods section 2.1.7). Reactions were incubated at 37°C for 20 minutes and then heat inactivated at 80°C for 5 minutes.

Table 2. Restriction enzyme DNA digest reaction volumes.

Reagent	Genomic/Plasmid DNA	PCR product
DNA	2µl (~1ug)	10µl (~500ng)
Fastdigest® Green buffer	2µl	2µl
Fastdigest® Enzyme	1µl	1µl
H_2O	15µl	17µl
Total reaction volume	***20µl***	***30µl***

2.1.3 Ligations

Following successful digestion of plasmid and genomic DNA for cloning, the vector and gene insert were ligated at a 1:3 molar ratio using 1 unit of T4 DNA Ligase (ThermoFisher Scientific, catalogue number EL0011) with the designated buffer (ThermoFisher Scientific, catalogue number B69) and H_2O, in a total volume of 10µl. A vector only control reaction was also set up to determine the presence of any undigested or re-ligated vector DNA. The reaction was incubated at room temperature overnight before being transformed (Methods section 2.1.3).

2.1.4 Transformation

Transformation of plasmid DNA was carried out using Subcloning Efficiency™ DH5α™ Chemically Competent Cells (ThermoFisher Scientific, catalogue number 18265017). A 25µl aliquot of these *E. coli* cells was thawed on ice for 20 minutes before adding ~10ng of

DNA. After ligation, 2.5µl of the ligation reaction was added to the cells and mixed gently with end of pipette tip. After incubating on ice for 20 minutes, the cells were heat shocked in a water bath at 42°C for 20 seconds, then immediately placed on ice for 2 minutes. Nutrient-rich SOC media (200µl) (Invitrogen™, catalogue number 15544034) was added to the cells for maximum transformation efficiency before placing in a shaking incubator for one hour at 37°C with 225 revolutions per minute (rpm). LB broth with agar (Sigma, catalogue number L3147) plates were made following manufacturer guidelines by adding 16g into 400ml of ddH_2O. Ampicillin was also added to the agar plates at 100µg/ml. All 200µl of the SOC, containing transformed cells, was plated and incubated at 37°C overnight.

The following afternoon, colonies were picked and inoculated in 6ml of LB broth growth media (ThermoFisher Scientific, catalogue number BP1426) containing ampicillin at 100ug/ml, to grow overnight (~16-18 hours) at 37°C and 225 revolutions per minute (rpm). Plasmid DNA was then extracted from the bacterial cells the following day (Methods section 2.1.6).

2.1.5 Colony Polymerase Chain Reaction (PCR)

If there were more than 50 colonies present post-ligation, and particularly in cases where the vector-only control indicated the presence of undigested/re-ligated vector, a colony PCR was carried out to identify positive clones with the correct size insert. Primers designed by Nigel Temperton (VPU, Kent) were utilised and synthesised by Eurofins Genomics (sequences below). The primers facilitated amplification of the DNA region bounded by the arms of the pI.18 vector, for example the gene cloned within the MCS.

pI.18 Forward primer 5’ GGT GGA GGG CAG TGT AGT CT 3’

pI.18 Reverse primer 5’ GCG AGG ATG TCA CCT GAT GG 3’

Individual colonies were picked with a sterile pipette tip and left in ddH_2O for 10 minutes before heating at 94°C for 3 minutes in a thermal cycler to burst-open the bacterial cells. Once cooled to room temperature 5µl of the lysed cell mix was added to a PCR reaction mix, which consisted of; 12.5µl of DreamTaq Green PCR Master Mix (2X) (ThermoFisher

Scientific, catalogue number K1081), 0.1µl FWD primer (0.2µM), 0.1µl REV primer (0.2µM) and 7.3µl ddH_2O. The DNA mixtures were placed in a thermal cycler and the following program run; 94^oC for 2 minutes before commencing 30 cycles of 94^oC for 30 seconds, 51^oC for 1 minute, 72^oC for 1 minute (allowing 30 seconds per Kb for extension) and then finally holding at 72^oC for 5 minutes. The resulting PCR products were analysed on an agarose gel (Methods section 2.1.7) to identify clones with the correct size insert (loading dye was not required due to presence of optimised Green dye within the DreamTaq Green PCR Master Mix). From these results, the respective colonies were re-picked and grown overnight in 6ml of LB broth growth media (ThermoFisher Scientific, catalogue number BP1426) for 16-18 hours at 37^oC and 225 revolutions per minute (rpm).

2.1.6 Plasmid DNA purification

The extraction and purification of plasmid DNA from transformed bacterial cells (Subcloning Efficiency™ DH5α™ Competent Cells from ThermoFisher Scientific, catalogue number 18265017) was carried out using a commercially available kit, the QIAprep Spin Miniprep Kit (QIAGEN, catalogue number 27104). Manufacturer's guidelines were followed with a final elution of the DNA in 30µl of H_2O at 70^oc to maximise the DNA yield. For DNA extraction from an agarose gel a commercially available QIAGEN kit was also used (QIAGEN, catalogue number 28704).

2.1.7 Measuring the concentration of DNA

The concentration and purity of DNA was determined by ultraviolet spectrophotometry at an absorbance ratio of 260/280nm using the NanoDrop™ 2000 Spectrophotometer (NanoDrop™ Products, ThermoFisher Scientific). Manufacturer instructions were adhered to, blanking with water.

2.1.8 Gel electrophoresis

To visualise DNA under ultraviolet light, DNA reactions were loaded on to a 1% (w/v) agarose gel (Fisher Scientific, catalogue number BP1356) consisting of 0.5X Tris-Acetate-

Ethylenediaminetetraacetic acid (EDTA) buffer (TAE 50X stock solution from Fisher Scientific, catalogue number BP1332) with the addition of Nancy-520 fluorescent stain (Sigma, catalogue number 01494). When the addition of a loading dye was required 6X DNA Loading Dye was used (Thermo Fisher Scientific, catalogue number R0611).

The agarose gel was placed in an appropriately sized gel tank in 0.5X TAE buffer with a power supply of 90V (Consort, catalogue number EV231) for 30 minutes. Once the run was complete, a G:Box gel imager (Syngene, G:Box Chemi XT Imaging System) and GeneSnap software were used to acquire images of the DNA migration patterns within the gel.

2.1.9 Site directed mutagenesis (SDM)

Primers were designed using Agilent Technologies QuikChange™ Site-Directed Mutagenesis Kit primer design program (http://www.genomics.agilent.com/primerDesignProgram.jsp) and are detailed in the methods section of relevant chapters. The PCR was set up using Accuzyme™ mix (25µl), 140ng of template DNA, 125ng[1] of FWD and REV primers, adding H_2O to a final volume of 50µl. The DNA mixtures were placed in a thermal cycler and initially denatured at 98°C for 3 minutes, before commencing 25 cycles of denaturation at 98°C for 20 seconds, annealing at 60°C[2] for 15 seconds, extending the DNA at 72°C for 12 minutes[3] and then further extension at 72°C for 20 minutes to maximise full length products. A small volume of PCR product, 5µl, was run on a 1% (w/v) agarose gel to check for amplification by visualising bands of DNA (Methods section 2.1.7). If positive for amplification, the PCR product was purified using a Qiagen PCR purification kit (Methods section 2.1.5) and the concentration of the resulting DNA was measured (Methods section 2.1.6). At this point it was necessary to digest the DNA with *Dpn*1 enzyme to remove any remaining parental (methylated) DNA; 700ng of DNA was digested with 1µl of fast digest *Dpn*1 enzyme, 1µl of fast digest buffer (ThermoFisher, catalogue number FD1703) and H_2O

[1] Quantity of primers required should be calculated using Agilent QuikChange™ formula:
X pmoles of primer = (125ng of primer / 330 x number of bases in primer) x 1000

[2]Annealing temperature to be defined according to primer design.

[3]Extension time to be determined by length of DNA: 2 minutes per Kb.

for a total reaction volume of 10µl. The digestion reaction was incubated for 20 minutes at 37^{o}C, followed by 80^{o}C for 5 minutes to deactivate the enzyme.

The resulting plasmid DNA was transformed into DH5α sub cloning efficiency *E. coli* competent cells and plated onto agar plates containing ampicillin (100µg/ml) overnight (Methods section 2.1.3). After successful transformation, two colonies were picked and overnight LB cultures were prepared. Plasmid DNA was purified from the bacterial cells using a Qiagen miniprep kit (Methods section 2.1.5), eluting in 50µl of H_2O at 70^{o}C and was sent for sequencing at GATC Biotech (Methods section 2.1.9).

2.1.10 Sequencing

To verify that the correct DNA sequence was inserted into pI.18 during cloning or to ensure the correct change had been incorporated during SDM, the recombinant DNA was sent for sequencing. Sanger sequencing via GATC Biotech was carried out by the LightRUN system, using the pI.18 primers detailed above (Methods section 2.1.4).

2.2 Cell culture

2.2.1 Cell lines and maintenance

Human embryonic kidney (HEK) 293T/17 cells were purchased from ATCC® (catalogue number CRL-11268™) for use as a producer and target cell line. The A72 cell line originated from a tumour of canine thigh tissue and was a kind gift from Dr Pablo Murcia (Centre for Virus Research, Glasgow). Both cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM PAN Biotech, catalogue number P04-04510) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS PAN Biotech, catalogue number P40-37500HI) and 1% (v/v) 1mg/ml penicillin-streptomycin (Sigma, catalogue number P4333), together referred to as D-10. Cells were grown on Nunclon® surface petri dishes (Sigma, catalogue number Z763632) at 37°C in 5% CO_2. To sub-culture the cells, D-10 was removed from the plate and trypsin EDTA solution (Sigma, catalogue number T3924) was added to detach cells from the surface of the plate. Cells were then resuspended in fresh D-10 and seeded at an appropriate density to maintain the cells.

2.2.2 Freezing and thawing cell lines

At 80% confluence, cells were trypsinised and resuspended in D-10 (three times the volume of trypsin) before centrifuging for 5 minutes at 500rpm. The pellet of cells from one 10cm Nunclon® dish was gently resuspended in a mixture of D-10 with 10% (v/v) dimethyl sulfoxide (DMSO from VWR International Ltd, catalogue number 282164K) in a total volume of 2ml. To freeze, 1ml aliquots in cryovials were stored in a Mr Frosty™ for 24 hours at -80°C before transferring to a storage box at -80°C.

To thaw an aliquot of cells, the cryovial was placed in a 37°C water bath for approximately 5 minutes (caution was taken not to allow the cells to reach 37°C to prevent DMSO toxicity affecting the health of the cells). The cells were resuspended in D-10 and centrifuged at 1000g for 5 minutes to remove the freezing media and finally resuspended in D-10 to be seeded on a 10cm Nunclon® dish.

2.3 Influenza lentiviral pseudotype virus (PV) protocol

2.3.1 PV production

2.3.1.1 Haemagglutinin (HA) only PVs

HA-only PVs were generated via a four plasmid co-transfection of HEK293T/17 cells using polyethylenimine (PEI) transfection reagent (Sigma, catalogue number 408727) at 1mg/ml (Figure 10). The HA surface glycoprotein expressed by pI.18, p8.91-HIV gag-pol retroviral core, pCSFLW-firefly luciferase reporter gene and endoprotease expressing plasmid (to cleave the HA) were combined with OptiMEM™ (ThermoFisher, catalogue number 11058021), whilst a separate OptiMEM™-PEI mixture was left to incubate at room temperature for 5 minutes. The OptiMEM™-DNA mix was then added to the OptiMEM™-PEI and incubated at room temperature for 20 minutes. The quantity of DNA plasmids and volumes of OptiMEM™ and PEI used were dependent on the size of the cell culture vessel (Table 3). Following 20 minutes of incubation at room temperature, the transfection mix was added to the monolayer of cells (at 70-80% confluence) and incubated at 37°C for 24 hours. Recombinant neuraminidase from *Clostridium perfringens* (Sigma, catalogue number N2876) was added 24 hours post-transfection, to ensure exit of viral progeny from producer cells. The pseudotyped virus was harvested at 48 hours through 0.45µm filters and virus supernatant was stored at -80°C in appropriate sized aliquots.

2.3.1.2 Haemagglutinin and neuraminidase PVs

To generate PVs that display both HA and NA glycoproteins on their surface, an NA-expressing plasmid was added to the OptiMEM™-DNA mix instead of the exogenous NA addition 24 hours post-transfection (Table 3).

Table 3. Transfection reagents and the quantity required for transfection within different sized cell culture vessels.

Transfection reagent	Quantity of reagent	
	Nunc™ Nunclon™ Vita 6-Well Multidish	**Nunclon® 10cm dish**
PEI (1mg/ml)	17.5 µl	35 µl
OptiMEM™	100 µl	200 µl
pI.18-HA	500 ng	1 µg
p8.91-gag-pol	500 ng	1 µg
pCSFLW	1 µg	1.5 µg
Exogenous NA	50 µl	100 µl
pI.18-NA	125 ng	250 ng

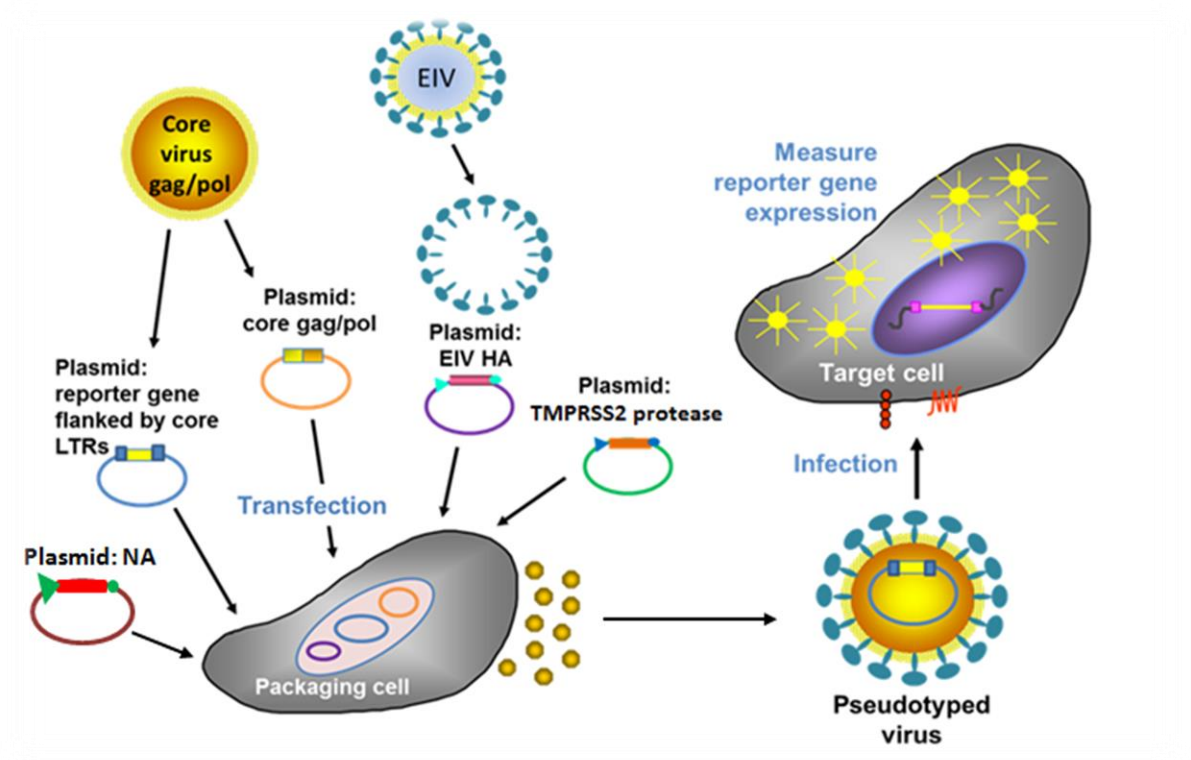


Figure 10. Schematic of four/five plasmid co-transfection system using HEK293T/17 cells (modified from Scott *et al*.2012) to generate pseudotype viruses.

2.3.2 PV titration

PVs were titrated using HEK293T/17 cells ($1x10^4$ cells/well) with an initial 100µl of neat virus-containing supernatant that was subsequently serially diluted 1:2 across a white 96-well plate (ThermoScientific™ Nunc™, catalogue number 136101) and incubated for 48 hours at 37°C. After 48 hours the cell cultures were assessed and luminescence read using the Bright-Glo assay system with a GloMax luminometer (Promega, UK). The PV titres are reported as relative luminescence units (RLU)/ml.

2.3.3 Pseudotyped virus neutralisation assay (PVNA)

Each serum sample was diluted 1/40 (unless otherwise stated in results chapters) with subsequent two-fold dilutions in a 96-well plate, using D-10 media. A PV input of $1x10^6$ RLU was added per well and then incubated for one hour at 37°C to allow neutralisation to proceed. HEK293T/17 cells ($1x10^4$ cells/well) were added to the serum plus PV combination and incubated for a further 48 hours at 37°C. Luminescence was measured following the same procedure as the PV titration (Methods section 2.3.2). PV only (no neutralisation) and cell only (equivalent to complete neutralisation) controls were included on each 96-well plate.

2.3.4 Statistical analysis

For analysis of PVNAs, the raw RLU data points were converted to a percentage neutralisation value, whereby 100% neutralisation equals the mean cell-only RLU value and 0% neutralisation equals the mean PV-only RLU value. The normalised data was then plotted on a neutralisation percentage scale and an IC_{50} value calculated, using the non-linear regression analysis on GraphPad Prism (Figure 11). The IC_{50} is the reciprocal of the serum dilution that induces 50% neutralisation.

$$\mathbf{Y = 100 / (1+10 \wedge ((LogIC_{50} - X) * HillSlope))}$$

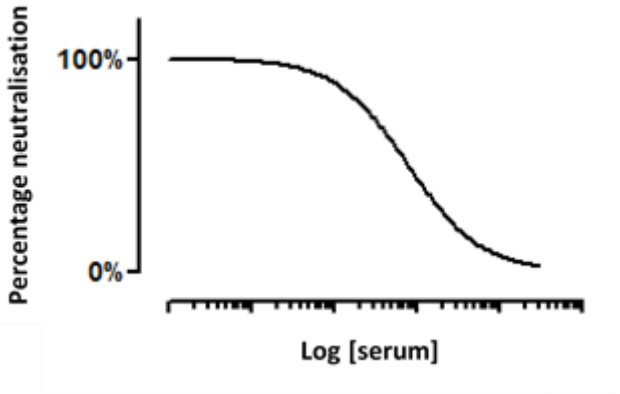


Figure 11. Example of GraphPad equation: log(inhibitor) vs. normalized response -- Variable slope – model used to fit curve for neutralisation data.

Each combination of serum and PV was tested at least in duplicate to calculate a mean IC_{50}. All IC_{50} values within the serum dilution range of the assay, were used to calculate a mean IC_{50}. Following this, fold-differences were calculated between different PVs assayed against the same serum sample. Negative values indicate a decrease in the IC_{50}.

2.4 Influenza MUNANA neuraminidase activity assay

The influenza 20-(4-methylumbelliferyl)-a-D-N-acetylneuraminic acid (MUNANA) neuraminidase activity assay protocol was edited from SOP V-6815/01-10 Angie Lackenby, Respiratory Virus Unit, Health Protection Agency (Wetherall *et al.*, 2003).

Table 4. Influenza MUNANA neuraminidase activity assay reagents.

Master stock solutions	
1) 325mM MES:	31.72g MES in 500ml ddH_2O (pH to 6.5 with concentrated NaOH)
2) 100mM $CaCl_2$:	5.55g CaCl2 in 500ml ddH_2O
3) 1M Glycine:	37.5g in 500ml ddH_2O (pH to 10.7 with concentrated NaOH)
4) 1mM MUNANA:	25mg in 51ml MES assay buffer (store -20^{O}C)
Working solutions and buffers	
5) MES assay buffer:	32.5mM MES (50ml solution **1**) 4mM $CaCl_2$ (20ml solution **2**) ddH_2O 430ml pH to 6.5 with concentrated NaOH
6) 100µM MUNANA (per plate):	300µl of solution **4** 2.7ml of solution **5**
7) Stop solution (500ml):	25% ethanol (125ml absolute ethanol) ddH_2O 325ml pH to 10.7 with concentrated NaOH

MES assay buffer was added to each well of black 96-well flat bottomed plate (20µl). PVs were then added and mixed well by pipetting, in duplicate, at a starting dilution of 1/2. A two-fold serial dilution was carried-out, leaving the final row as a negative control, containing the buffer only. The MUNANA substrate (working solution 5; Table 4) was prepared with 3ml for each plate and 30µl added to each well, including the blank row. The plates were sealed, wrapped in foil and placed in a shaking incubator, at 37^{o}C, for one hour. To terminate the reaction, 150µl of solution7 (Table 10) was added to all wells. The plate was read within 30 minutes of adding the stop solution with the following settings; excitation 355 and emission 460.

3 EQUINE INFLUENZA PSEUDOTYPE VIRUS PRODUCTION AND OPTIMISATION

3.1 Introduction

Optimisation of pseudotype virus (PV) production protocols is necessary to ensure efficient generation of high-titre PVs. High-titre PV preparations are desirable as this reduces the need to use multiple batches during large-scale studies. A PV 'batch' consists of the total volume of PV-containing cell culture supernatant that is collected and pooled (if necessary) on the same day. There are several aspects to the production of PVs that can be optimised but the haemagglutinin (HA) and neuraminidase (NA) surface glycoproteins form the main focus of this chapter. Specifically, the cleavage of HA and the source of NA.

The HA protein is fundamental for influenza virion binding to sialic acid-containing receptors on a target cell. A prerequisite for successful infection is cleavage of the HA precursor (HA0) into its respective HA1 and HA2 subunits, which can occur intra- or extra-cellularly (Bertram et al., 2010). Once a virion is endocytosed, the cleaved form of HA undergoes a conformational change triggered by a low pH within the endosome. The change in protein structure exposes a fusion peptide that facilitates fusion of the virion with the endosomal membrane (reviewed in Skehel and Wiley, 2000). The fusion event is essential for release of viral RNA into the cytosol, which can then be used for replication of the viral genome.

Cleavage recognition sites differ between strains of influenza; polybasic cleavage sites within highly pathogenic influenza strains (H5 and H7) can be cleaved by non tissue-specific proteases such as furin whereas low pathogenic variants (for example the H3 strains used in this study) require cell specific protease activity. Therefore, for the production of an influenza pseudotyped virus with a monobasic cleavage site, the correct choice of protease is essential. Type II transmembrane serine proteases TMPRSS2 (transmembrane protease serine 2), TMPRSS4 and HAT (human airway trypsin-like protease) are known to cleave HA of human influenza viruses (Böttcher *et al.*, 2006). TMPRSS2 has been shown to effectively cleave group two human HAs (subtypes H3, H4, H7, H10, H14 and H15) (Ferrara *et al.*, 2012) as well as an equine H3 HA (Scott *et al.*, 2012). However, the optimisation and efficacy of potential alternatives such as TMPRSS4 and HAT, have only been tested for human H3 PVs (Bertram et al., 2010).

In nature, influenza NA cleaves sialic acid receptors permitting viral egress. The NA enzyme is therefore essential for efficient influenza PV production (Cheresiz *et al.*, 2014). Both the source and quantity of NA can be altered during the production of PVs. An exogenous source of NA (often from *Clostridium perfringens*) is typically added for cleavage (Scott *et al.*, 2012). However, an endogenous source of NA (i.e. NA-encoding plasmid) has also been trialled alongside different HA subtypes (e.g. H5 with N1 (Cheresiz *et al.*, 2014) and H7 with N1, N3 and N7 (Molesti *et al.*, 2012). This results in NA being expressed on the pseudotype virus envelope. The titres of PVs incorporating both HA and NA have been shown to vary. One study showed that the utilisation of an N2 plasmid, compared to an exogenous source of NA, increased the titre of a human H3 PV (Ferrara, 2016). Similarly, co-transfection of matched H1 and N1 avian glycoproteins yielded a higher PV titre than an exogenous source of NA (Molesti *et al.*, 2012). Therefore, the addition of an NA plasmid may be useful to increase PV titre in some instances.

The aim of this chapter was to optimise the production of equine influenza PVs, first through trialling different types and quantities of protease plasmid and secondly, by adding an endogenous NA plasmid in contrast to exogenous NA during PV production.

3.2 Materials and methods

3.2.1 Plasmids

A/equine/Newmarket/1979 (H3) (Nm79) and A/equine/Richmond/1/2007 (H3) (Rich07) PCR products, amplified to include restriction enzyme (RE) recognition sites for *EcoRV*/*BamH*I and *BamH*I/*Xho*I at 5'/3' terminii (enzymes from Thermo Scientific) respectively, were kindly provided by Dr Adam Rash and Dr Debra Elton at the Animal Health Trust. These H3 genes were cloned into the mammalian expression plasmid pI.18 (see Chapter 2 Methods section 2.1.2) for PV production at the University of Kent.

The N8 (A/equine/Richmond/1/2007 (H3N8), GenBank: KF559336.1) gene sequence was synthesised with terminal *BamH*I/*Xho*I sites incorporated for cloning into pUC57 by GenScript®. The lyophilised pellet of this pUC57 N8 equine recombinant plasmid was resuspended in 20µl of ddH_2O (manufacturer guidelines) and re-transformed into

chemically competent Subcloning Efficiency™ DH5α™ Competent Cells (Chapter 2 Methods section 2.1.4). The gene was then subcloned into plasmid vector pI.18 (Chapter 2 Methods section 2.1.2).

The plasmids expressing the pCAGGS-TMPRSS2 and pCAGGS-HAT endoproteases were kindly provided by Dr Eva Böttcher and Professor Hans Dieter Klenk, and pCMV-Tag3-TMPRSS4 was gifted by Professor Stefan Pöhlmann.

3.2.2 PV production

PVs displaying only HA were produced as described in (Chapter 2 Methods section 2.3.1.1). Briefly, HEK293T/17 cells were co-transfected (using transfection reagent PEI) with plasmids expressing an equine influenza H3 surface glycoprotein, HIV gag-pol, firefly luciferase reporter gene and endoprotease. All three proteases were tested using 125, 250, 500, 750 and 1000ng of expression plasmid DNA.

After protease optimisation, optimal protease conditions were used in a five p

lasmid co-transfection protocol, with the addition of 125ng of NA plasmid (4:1 ratio of HA:NA). All PV titrations were performed as described previously and titres calculated in RLU/ml (Chapter 2 Methods section 2.3.2).

3.2.3 Statistical analysis

Comparison of two data sets was carried out using an unpaired T-test, except in cases where a normal distribution could not be assumed. In such cases, the Mann-Whitney test was applied. Values outside of the 95% confidence limits were deemed to be statistically significant. GraphPad Prism version 5 software was used for analysis.

3.3 Results

3.3.1 Cloning of Nm79 and Rich07 HAs

The Nm79 HA PCR product was amplified with primers incorporating *BamH*I and *Xho*I sites and ligated into pI.18, which was digested with the same enzymes. Post transformation, three colonies were picked, cultured overnight and the plasmids purified (Chapter 2 Methods section 2.1.4). The plasmids were screened by restriction enzyme digest, to determine if the ligation had been successful. One positive colony was identified, however the gene was truncated at ~1100bp not the expected 1700bp for HA (Figure 12). The full Nm79 sequence was checked for restriction sites and a *BamH*I site was identified between bases 587–592, confirming the cause of the truncated DNA (Figure 13).

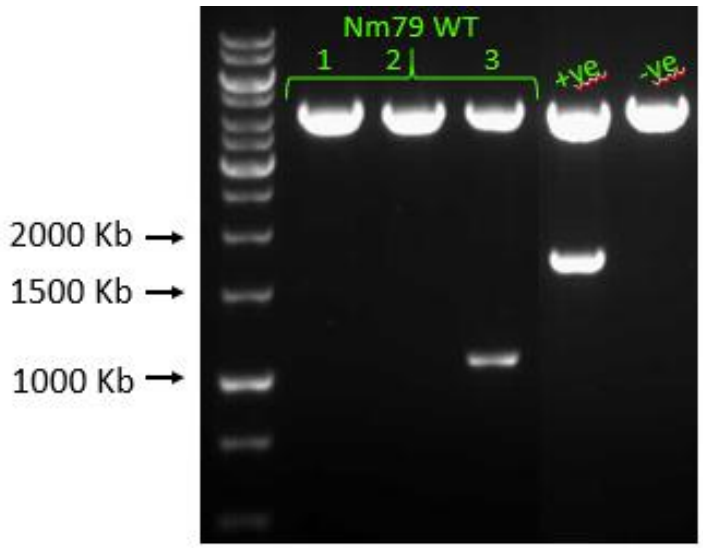


Figure 12. Digest screen of A/equine/Newmarket/1979 HA in pI.18 (1-3) using *Kpn*I and *Xho*I REs. All three clones were negative for the correct size insert. Positive control (+ve) was A/equine/Newmarket/5/2003 HA in pI.18 and negative control (-ve) was pI.18 digested with *Kpn*I and *Xho*I.

```
        430                                                        480
acatggacag gtgtcactca aaacggaaga agtggcgcct gcagaagggg atcagccgat

        490                                                        540
agtttcttta gccgactgaa ttggctaaca aaatctggag attcttaccc cacattgaat

        550                                              590       600
gtgacaatgc ctaacaataa caatttcgat aaactataca tctgggggat ccatcacccg
```

Figure 13. Section of A/equine/Newmarket/1979 HA nucleotide sequence with *BamH*I restriction site (GGATCC) highlighted at positions 587–592.

In order to address this truncation issue, a blunt end cloning technique was adopted. The Nm79 HA PCR product was digested with *Xho*I (3' end) and pI.18 was digested sequentially with *EcoRV* and *Xho*I, facilitating the 3' directional/5' blunt end cloning strategy. After ligating the inserts into pI.18 and transforming them, a colony PCR was carried out, to identify colonies containing the correct size insert at ~1700bp. Eight colonies were picked and five out of the eight were positive for 1700bp inserts (Figure 14). Sequence analysis confirmed the positive clones to be Nm79 HA.

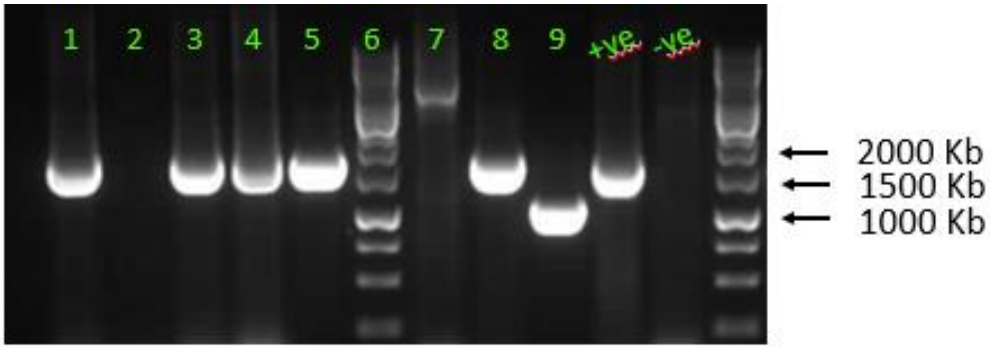


Figure 14. Colony PCR of A/equine/Newmarket/1979 HA insert in pI.18. Lanes 1, 3, 4, 5 and 8 were positive for the correct size insert at 1.7Kb. Lanes 2, 7 and 9 were negative for HA. Positive control (+ve) was A/equine/Sussex/1989 HA in pI.18 and negative control (-ve) was pI.18 cut with *BamH*I and *Xho*I.

Primers designed to amplify Rich07 cDNA also incorporated *BamH*I and *Xho*I sites, neither of which appear within the HA sequence of this strain. Cloning was carried out without any unforeseen digestion issues and a colony PCR of ten colonies identified one positive clone (Figure 15). The positive colony was cultured, purified, confirmed positive by RE digest and sequenced before being used in transfections.

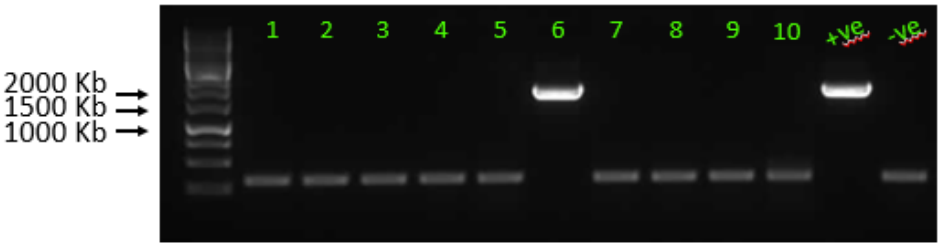


Figure 15. Colony PCR of A/equine/Richmond/1/2007 HA insert in pI.18. Lane 6 contains a positive clone, whereas 1–5 and 7–10 appeared negative. Positive control (+ve) was A/equine/Newmarket/1979 HA in pI.18 and negative control (-ve) was pI.18 cut with *BamH*I and *Xho*I.

3.3.2 Protease dependent generation of Nm79 and Rich07 PVs

The newly cloned Nm79 and Rich07 HA-pI.18 plasmids were transfected alongside HIV gag-pol, firefly luciferase and various protease plasmids at different concentrations into HEK293T/17 cells. Two controls were incorporated; delta protease (HA but no protease) and delta env (no HA envelope protein or protease). The former indicates luminescence attributed to HA-mediated entry without specific protease activity. The latter indicates background luminescence not attributed to glycoprotein-specific mediated entry. It is evident that PV titre does differ, depending on the quantity and type of protease plasmid used (Figure 16). The titres of Rich07 with TMPRSS2 and HAT differed significantly when 125 and 1000ng were compared (unpaired T-test; $p = 0.016$ and $p = 0.023$, respectively). This was also the case for Nm79 ($p = 0.010$ and $p = 0.008$, respectively). Interestingly, the smallest quantity of TMPRSS2 and HAT protease expression plasmids (125ng) generated the highest titres. However, for TMPRSS4 the titres were not significantly different (Nm79 $p = 0.198$ and Rich07 $p = 0.325$) from the delta protease, implying that it does not effectively cleave either of the equine H3 HAs. Cleavage of the HA using 125ng of TMPRSS2 for both Nm79 and Rich07 significantly increased PV titre compared to delta envelope ($p = 0.004$ and $p = 0.009$ respectively), likewise with HAT ($p = 0.004$ and $p = 0.020$). For Nm79 there was no significant improvement in titre between TMPRSS2 and HAT ($p = 0.217$) but for Rich07, HAT was advantageous ($p = 0.042$).

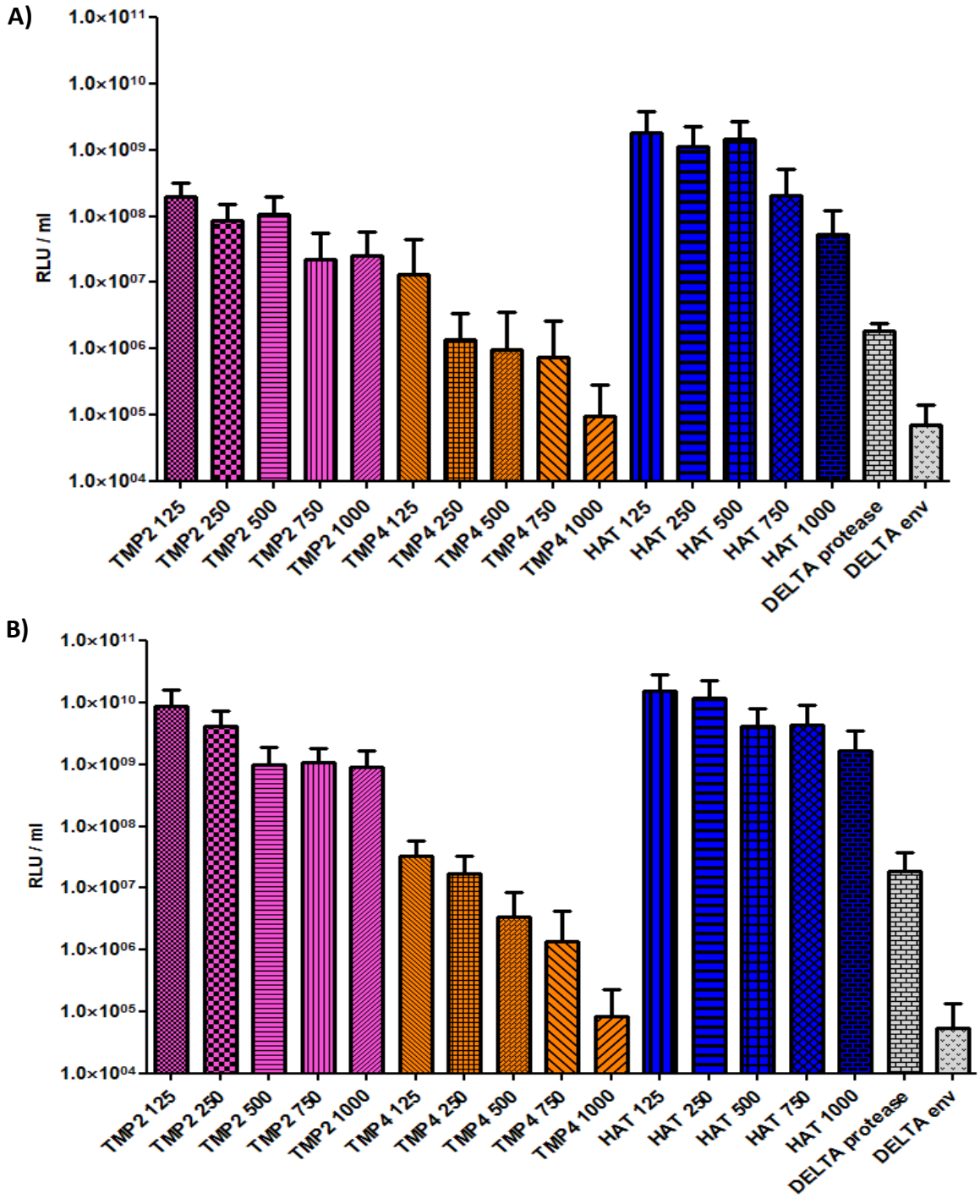


Figure 16. PV titres obtained with **A)** A/equine/Richmond/1/2007 H3 and **B)** A/equine/Newmarket/1979 H3 in combination with selected protease-expressing plasmids. TMPRSS2 (TMP2), TMPRSS4 (TMP4) and HAT proteases were trialled, each number denotes the quantity of protease plasmid used (ng). PV titres reported in relative luminescent units (RLU/ml) and error bars represent +SEM from 8 replicates.

3.3.3 Sub-cloning of Rich07 N8 gene

Transformation of *E. coli* host cells with pUC57-N8 was successful. The plasmid-containing *E. coli* cells were then cultured and the plasmids purified. The N8 gene was then excised from pUC57 using *BamH*I/*Xho*I REs: Gel electrophoresis confirmed that the digestion was successful (gel photo not shown) and subsequently the DNA was extracted from the gel and purified (Chapter 2 Methods section 2.1.6). Plasmid vector pI.18 was digested with the same REs (*BamH*I/*Xho*I) and ligated with the N8 gene. Ligations were set up at a 1:3 molar ratio of vector: insert (plus a vector only control). Post transformation, more than 1000 colonies were obtained for pI.18-N8. A smaller volume of bacterial culture was plated and re-plated until individual colonies were discreet. A colony PCR identified that all colonies were negative for the insert. Therefore, a new plasmid stock of pI.18, digested with *BamH*I and *Xho*I was prepared and resulted in a much higher concentration of DNA with a 260/280 ratio of 1.85. Using the new plasmid stock, ligations with N8 were set up at 1:1 and 1:3 ratio of vector: insert. Transformations were successful and ten colonies were carried forward into a colony PCR. One positive clone was identified at the 1:3 ratio (Figure 17).

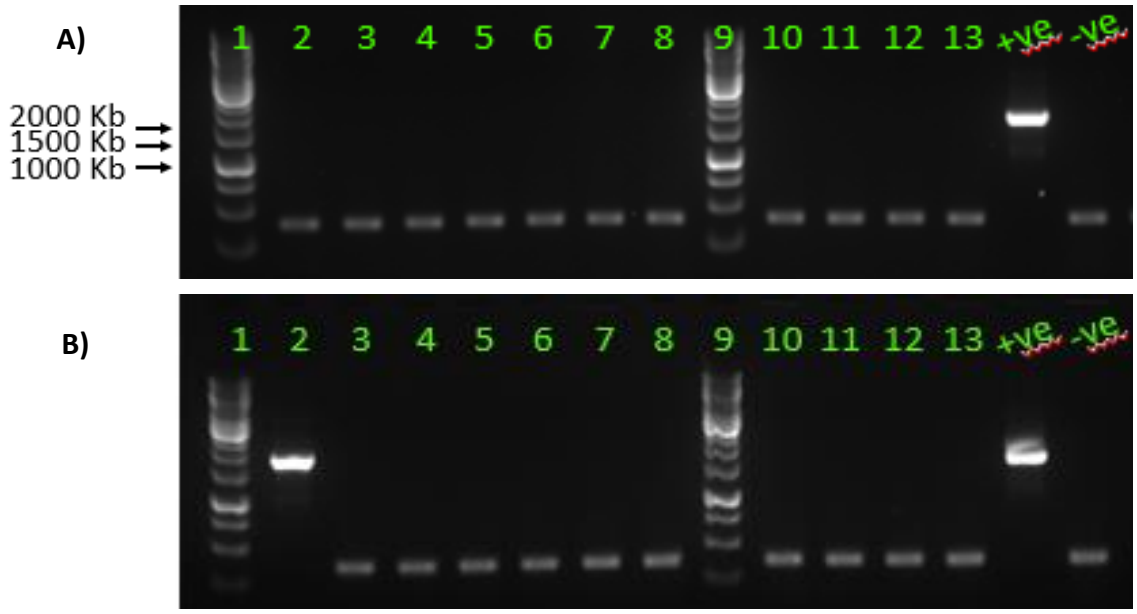


Figure 17. Colony PCR of pI.18-N8 equine (from A/equine/Richmond/1/2007 H3N8). Lanes 2-13 (**A**) are 1:1 molar ratio ligations, all negative for N8 gene. Lanes 2-13 (**B**) are 1:3 molar ratio ligations, with a positive clone in lane 2. Positive control (+ve) is A/chicken/Italy/1067/1999 N1 in pI.18 and negative control (-ve) was pI.18 digested with *BamH*I/*Xho*I.

3.3.4 Equine H3 and N8 PV

In order for a PV preparation to be considered functional, the difference in RLU/ml between Δenv negative control (no HA or NA present) and the PV, should be significant, as particles have been demonstrated to enter target cells via the surface envelope glycoprotein. The addition of exogenous NA to generate an H3 only A/equine/Richmond/1/2007 PV yielded a titre much greater than Δenv ($p < 0.001$). The co-transfection of H3 and N8 plasmids to produce Rich07 H3N8 PV, was also successful ($p < 0.001$), however the titre was not as high as using exogenous NA (Figure 18). As expected, there was no difference in RLU/ml when the H3ΔNA control and Δenv were compared ($p = 0.074$), highlighting the necessary presence of NA for PV release.

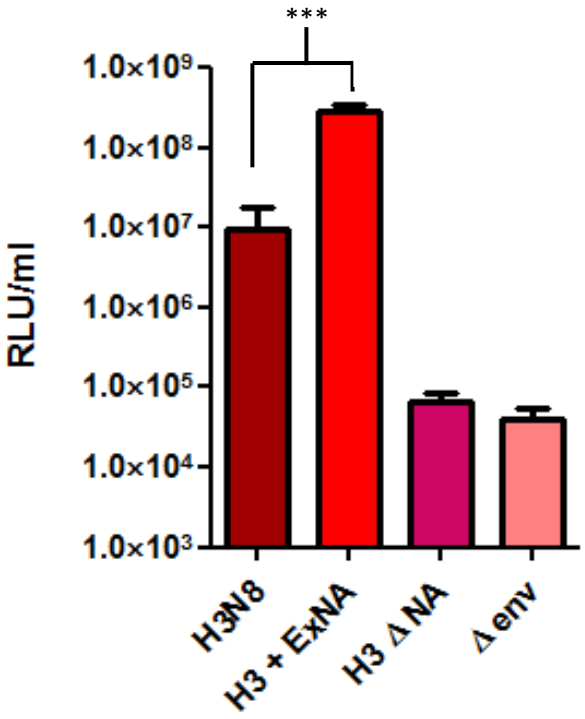


Figure 18. A/equine/Richmond/1/2007 H3 PV titres of preparations generated by co-transfection with an N8 encoding plasmid or using the standard technique of adding exogenous (Ex) NA (positive control). H3ΔNA (H3 without NA) and Δenv (no HA or NA) were generated as negative controls. Average PV titres reported in relative luminescent units (RLU/ml), with error bars indicating +SEM, based on 8 replicates. The statistically significant difference between H3+ExNA and H3+N8 encoding plasmid is indicated by *** (Mann-Whitney test; $p < 0.001$).

3.4 Discussion

A/equine/Richmond/2007 and A/equine/Newmarket/79 H3 glycoproteins were effectively cleaved by TMPRSS2 and HAT proteases, facilitating PV production. In both cases, the smallest quantity of transfected protease plasmid (125ng) yielded the highest PV titre, implying that a greater quantity of protease does not enable more effective cleavage of the HA. TMPRSS2 is known to play an HA cleavage role in the activation of human H3 HA. A recent study also showed that TMPRSS2 knock-out mice experienced reduced weight-loss when infected with an H3N2 virus. However, survival was not improved, indicating the role of another protease (Hatesuer *et al.*, 2013). Interestingly, survival against a H3N2 virus was greatly improved for knock-out TMPRSS2 and TMPRSS4 mice, highlighting the role of TMPRSS4 in H3 cleavage and subsequent virus infectivity (Kühn *et al.*, 2016). By contrast, in the current study, TMPRSS4 did not activate the HA to facilitate the production of either equine H3 PVs. Similarly, to TMPRSS2 and HAT, 125ng of TMPRSS4 plasmid yielded the highest PV titre, however this was not significantly different from the delta protease controls (Nm79 p = 0.198 and Rich07 p = 0.325). TMPRSS4 has been shown to effectively cleave H3s during PV production (Ferrara, 2016), however, the H3s were of human not equine origin. The cleavage sites of the human H3s (A/Udorn/307/1972 and A/California/7/2004) used in that study and the equine H3s differ, which may explain the lack of cleavage. The human H3 cleavage sites contain a threonine, 'PEKQ**T**R/G' in the place of an isoleucine, 'PEKQ**I**R/G' for equine H3 (Table 5). The consensus sequence for the H3 HA cleavage site is PEKQTR, with cleavage occurring after the single arginine (Sun *et al.*, 2010). From the limited data available (Chaipan *et al.*, 2009; Ferrara, 2016), TMPRSS4 most effectively cleaves influenza HA sequences with a polar amino acid threonine/serine preceding the cleavage site (Table 5). Interestingly the 1918 'Spanish Flu' virus (H1N1), which claimed more than 50 million lives, contained a monobasic cleavage site. Chaipan et al. (2009) illustrated how TMPRSS4 successfully cleaves the Spanish H1N1, which also contains a polar amino acid before the cleavage site (serine).

Table 5. Comparison of cleavage sites from pseudotype viruses that have been successfully cleaved with TMPRSS4 (✓) and those that were not (**X**).

Influenza strain	Subtype	Cleavage site	Cleaved
H3 consensus	H3	P E K Q – T R	
A/South Carolina/1/1918	H1	. S I . – S .	✓
A/Korea/426/1968	H2	. Q I E – . .	✓
A/Udorn/307/1972	H3	. . . . – . .	✓
A/California/7/2004	H3	. . . . – . .	✓
A/Wisconsin/67/2005	H3	. . . . – . .	✓
A/equine/Richmond/1/07	**H3**	. . . . – I .	**X**
A/equine/Newmarket/1979	**H3**	. . . . – I .	**X**
A/duck/Czechoslovakia/1956	H4	. . . A – S .	✓
A/turkey/Ontario/6118/1968	H8	. S V E – P .	✓
A/chicken/Germany/N/1949	H10	. . V V Q G .	**X**
A/shearwater/West Australia/2576/79	H15	. . . I R T .	✓

It would be advantageous to be able to identify an optimal protease for HA cleavage during pseudotype virus production by sequence analysis, however, more data would be necessary to determine if this is a plausible approach. It has been shown that the matriptase protein (another member of the type II transmembrane serine family) cleaves some strains, though not all, within the H1 subtype (Hamilton *et al.*, 2012). This suggests using consensus sequences may not be accurate for determining the optimal protease to use. It might be appropriate to use a site directed mutagenesis approach to confirm specific amino acids that affect cleavage by particular proteases (Passero *et al.*, 2012). Site directed mutagenesis has already been trialled with respect to introducing a polybasic in place of a monobasic cleavage site, in an attempt to improve pseudotype production, but unfortunately it did not yield higher titre PVs as typically seen with polybasic H5 strains (Sawoo *et al.*, 2014).

An interesting finding is the effect of multiple proteases on the HA cleavage process. Different combinations of proteases have been shown to increase the

effectiveness of cleavage; TMPRSS2 with HAT (Sawoo *et al.*, 2014) and TMPRSS2 with TMPRSS4 (Kühn *et al.*, 2016). TMPRSS2 is known to cleave HA during transport to the host cell membrane of an infected cell, whereas HAT cleaves upon insertion to the cell membrane (Bertram *et al.*, 2010b). Therefore, any uncleaved HA that bypassed TMPRSS2 essentially has another opportunity to be cleaved by HAT. It is possible that proteases could be selected by sequence analysis and two proteases that act at different time points in the influenza life cycle utilised to provide increased opportunities for cleavage.

Studies have already shown it is possible to produce human and avian influenza PVs that express both HA and NA (Cheresiz *et al.*, 2014; Molesti *et al.*, 2012) but not thus far for equine PVs. Here, it is evident that an exogenous source of NA generates a higher titre PV than an endogenous source of NA but nevertheless the N8-encoding plasmid does facilitate the production of a functional PV. Neuraminidase antibodies are known to aid virus neutralisation (Marcelin *et al.*, 2012). Therefore, production of PVs incorporating both HA and NA could be beneficial for quantifying the additive effect of anti-NA antibodies, as well as anti-HA, in neutralisation assays. The functional H3N8 PV could be a useful tool for vaccine immunogenicity trials in the future. For studies that focus on anti-HA antibody responses, the HA-only PVs produced with exogenous NA are a more appropriate tool to dissect a specific response. Similarly, NA-only PVs would be useful for studying anti-NA antibodies, however a new approach to quantify these PVs would be necessary as the currently employed titration assay quantifies PVs based on virus particle entry rather than exit and without the HA, quantification would not be possible.

Optimising the titre of HA-only equine PVs will be useful for future investigations to reduce the likelihood of having to use different PV batches, which may potentially introduce an additional study variable. Furthermore, the production of HA+NA PVs will broaden the application of PVs as tools to study different aspects of equine influenza biology, specifically seroepidemiology and vaccine evaluation. It is also important to consider that working within strict budgets can be a challenge and thus investigating ways to reduce cost, without impeding the quality of data, may help such financial situations. Further to employing different HA and NA approaches in pseudotype production, it is possible to use different reporter gene/substrate systems that will further reduce the cost of pseudotyping and hopefully make reagents more accessible to laboratories in low-and-middle-income countries.

4 APPLICATION OF THE PSEUDOTYPED VIRUS NEUTRALISATION ASSAY TO EQUINE INFLUENZA VACCINE EFFICACY TESTING

4.1 Introduction

Equine influenza vaccines were made available in the UK in the late 1960s, shortly after the first human influenza vaccines. There are currently four different equine influenza A vaccines available in the UK (Animal Health Trust, 2017). For human influenza vaccines, a panel of experts meet annually to recommend the strains to be included in equine influenza vaccines. At present, Florida-sublineage clade 1 and 2 viruses continue to circulate in Europe while clade 1 predominates in America (Bryant *et al.*, 2011). The current recommendation is for strains representative of both clades to be included in vaccines (OIE, 2017).

Vaccine manufacturers have to conduct efficacy studies to assess the level of antibodies induced by vaccination and whether or not the vaccine provides protection against clinical signs after challenge with a representative virus. The serological assays currently employed to measure antibody responses against equine influenza vaccination are SRH and HI. Notably, these assays do not measure neutralising antibody responses and at present, there is not a serological assay readily available to measure neutralising antibody responses to equine influenza. The advantages and disadvantages of different serological assays for equine influenza are reviewed in Kinsley et al., 2016. As the PVNA has already been shown to distinguish between positive and negative equine influenza serum samples, neutralisation data would help to identify promising vaccine candidates and evaluate the neutralising antibody response from experimentally infected equines.

Assessments of intra-lab and inter-lab variability have been carried out for Single Radial Haemolysis (SRH) and Haemagglutination Inhibition (HI) assays but not thus far for the PVNA. The high variability in results across laboratories that is seen with HI and SRH has been well-documented for many years and still needs to be addressed (Daly *et al.*, 2007a; Wood *et al.*, 1994, 2011, 2012). Assays that can be used in laboratories within different institutions, with limited variability, will ultimately infuse more confidence in results, which could increase their adoption, for example in vaccine efficacy testing. Therefore, comparisons between traditional assays and investigations into the variation of newer assays such as the PVNA are important if they are to be considered for use in large

scale studies internationally. Comparative studies between the HI assay and the PVNA for human influenza have so far been carried out with promising results (Alberini *et al.*, 2009; Garcia & Lai, 2011; Yang *et al.*, 2014). Additionally, there is some evidence that the assay demonstrates greater sensitivity as some serum samples (from vaccinated animals) have been defined as negative by SRH but positive by PVNA (Scott *et al.*, 2012).

It is not always possible to produce large-enough quantities of pseudotyped virus (PV) at one time to complete a project. This may be because the particular pseudotype has a low titre and production in larger culture vessels is not as efficient, but also unplanned repeats may necessitate using different PV batches. Therefore, it would be beneficial to investigate variation between PV batches. Furthermore, the assessment of the reproducibility of the pseudotype virus neutralisation assay (PVNA) would give an insight into the robustness of the assay. This is essential if the assay were to be adopted by numerous laboratories and considered for use in a commercial setting. There are several variables that could affect the repeatability of the PVNA. The two primary variables are serum and PV; neat or pre-diluted sera can be used and batches of PV can vary in titre.

The aim of this study was to assess performance of PVNA in the context of a vaccine efficacy trial. Objectives were to (i) assess the reproducibility of the PVNA (ii) the capability of the assay to distinguish between homologous and heterologous PV and serum pairings (iii) employ PVNA in a vaccine efficacy trial to evaluate the neutralising antibody response to two different monovalent vaccines and to compare the results to those acquired through traditional methods such as SRH and HI.

4.2 Materials and methods

4.2.1 Serum samples

A positive H3 polyclonal equine serum sample was gifted by Dr Janet Daly for use in the repeatability studies. Post infection ferret sera against Nm79, Sx89, Nm/1/93, Nm/5/03 and SA/4/03 as well as post vaccination equine sera against Nm79, Sx89 and Rich07, containing subtype-specific antibodies, all kindly provided by Dr Adam Rash and Dr Debra Elton of the Animal Health Trust (AHT), were used in PVNAs where homologous/heterologous serum vs. PVs combinations were tested. During a vaccine efficacy trial, which was conducted by Dr Romain Paillot (Study Investigator) at the Animal

Health Trust for reasons unrelated to this thesis, serum samples were collected from Welsh mountain ponies housed at the AHT for use in PVNAs. Serum samples were collected at five time points (D0, D28, D56, pre-C and C+14) as described in Figure 19 from six groups of ponies, giving a total of 134 serum samples (Table 6).

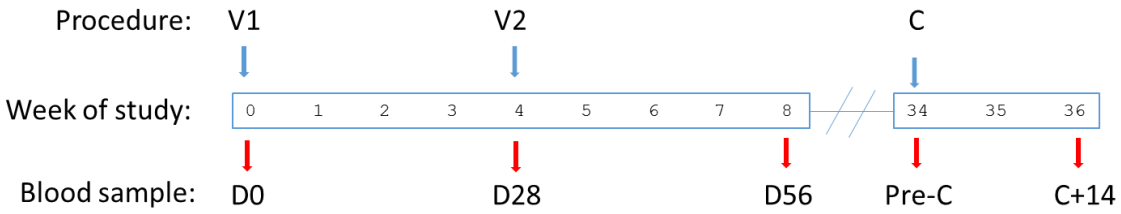


Figure 19. Timeline of study. V1, V2 = vaccination 1 and vaccination 2, C = challenge. Blood samples were collected at five time points; day 0 (D0), day 28 (D28), day 56 (D56), pre-challenge (Pre-C) and 14 days post challenge (C+14).

Table 6. Serum samples collected during the vaccine efficacy trial at the Animal Health Trust. The five ponies in groups 1–4 were administered a high or low dose of vaccine containing a Florida clade 2 (FC2) or a Florida clade 1 (FC1) strain. The FC1 and FC2 strains and the vaccine dose administered to groups 1–4 remain undisclosed for vaccine manufacturer confidentiality reasons. The 4 ponies in group 5 served as unvaccinated controls to confirm infectivity of the FC2 challenge virus. The 3 ponies in group 6 were administered a commercial EI vaccine (canarypox-based EI vaccine; ProteqFlu) containing an FC1 and an FC2 strain (FC1+FC2).

Group	n (horses)	n (samples)	Vaccination	Challenge
1	5	25	FC2 (high dose)	FC2
2	5	25	FC2 (low dose)	FC2
3	5	25	FC1 (high dose)	FC2
4	5	25	FC1 (low dose)	FC2
5	4	20	None	FC2
6	3	14	FC1+FC2	FC2

4.2.2 Pseudotype virus production and titration

A/equine/Newmarket/1979 (H3) (Nm79) and A/equine/Richmond/1/2007 (H3) (Rich07) HA gene PCR products, amplified with primers containing *EcoRV*/*BamHI* and *BamHI*/*XhoI* restriction sites at their 5' and 3' termini, respectively, were kindly provided by Dr Adam Rash and Dr Debra Elton at the Animal Health Trust. The H3 genes were cloned into the mammalian expression plasmid pI.18 and PV produced, by co-transfection (using transfection reagent PEI) of the equine influenza H3 surface glycoprotein alongside HIV gag-pol, firefly luciferase reporter gene and endoprotease pCAGGS-HAT (kindly donated by Dr Eva Böttcher, Philipps University Marburg) at the University of Kent. Two batches of the Nm79 PV were produced 11 months apart and stored at -80^{o}C to test the variation of PVNAs associated with different batches of PV. All PV titrations were performed as described previously and titres calculated in RLU/ml (Chapter 2 Methods section 2.3.2).

4.2.3 Serological assays

PVNAs were performed as described in (Chapter 2 Methods section 2.3.3). Briefly, serum samples were serially diluted and mixed with PVs for 1hr at 37°C. HEK293T/17 cells were then added and the reagents incubated for 48hrs. Luciferase expression was quantified using BrightGlo reagent (Promega). HI and SRH assays were performed at the Animal Health Trust.

4.2.4 Statistical analysis

IC_{50} values were calculated using GraphPad Prism software and repeat values were averaged using a mean. Statistical tests such as the paired T-test, one-way ANOVA and correlation analysis were also carried out using GraphPad Prism software.

4.3 Results

4.3.1 Reproducibility of PVNA

To analyse the reproducibility of the PVNA, the equine H3 positive serum (Methods section 4.2.1) was tested in quadruplicate, without pre-dilution, using a single batch of PV. The assays were conducted within a 24-hour period, using the same passage of cells, to establish the consistency of IC_{50} readings (Figure 20). The IC_{50} values from each of the four groups were compared and were found not to exhibit significant differences (comparison via one-way ANOVA: $p = 0.318$).

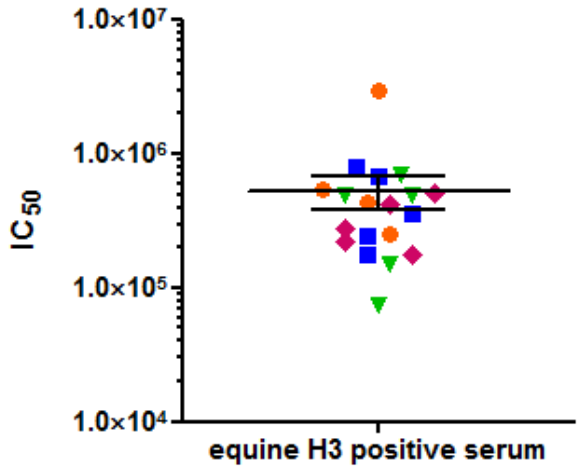


Figure 20. IC_{50} values calculated for an equine H3 positive control serum tested in quadruplicate (or quintuplicate) against A/equine/Richmond/1/2007 H3 PV in four independent assay plates (the four coloured symbols indicate data from each test repeat). Diluted serum was added to the assay to give a starting dilution of 1/800. Line with error bars represents mean with SEM.

Depending on the size of a study, it may not be possible to carry out all assays at the same time. To investigate the impact of increasing time between testing on data reproducibility, five independent PVNA assays were conducted at a minimum interval of one week. Again, there were no significant differences in the IC_{50} values between assays (Figure 21; $p = 0.144$).

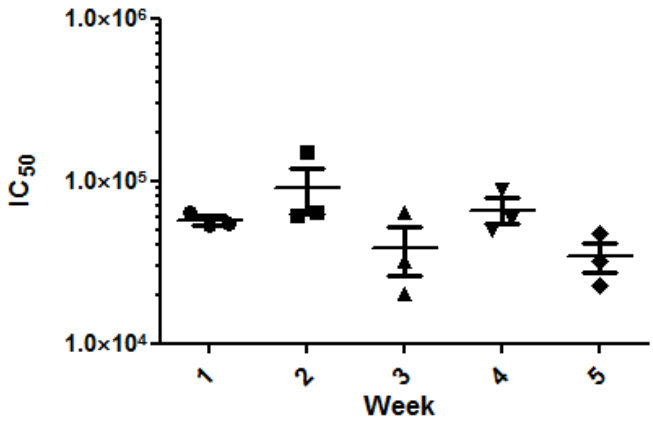


Figure 21. IC_{50} values calculated for an equine H3 positive control serum tested in triplicate against A/equine/Newmarket/1979 PV H3 PV at weekly intervals. Neat serum was added to the assay for a starting dilution of 1/40. Line with error bars represents mean with SEM.

The next stage was to compare different batches of PVs. PV batch 1 gave consistent IC_{50} results compared to PV batch 2 (Figure 22). However, comparing the combined data points for batch 1 to batch 2 showed no significant difference (unpaired T-test; $p = 0.094$).

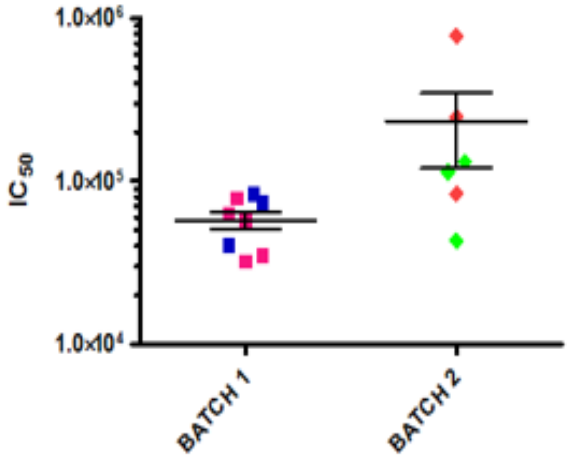


Figure 22. Comparison of IC_{50} values for two different batches of PV A/equine/Newmarket/1979 PV H3. Line with error bars represents mean with SEM.

Adding neat serum into the assay, instead of pre-diluting to spare serum stocks, reduced the IC_{50} but not significantly (Figure 23; $p = 0.163$). These data imply that it is acceptable to use different batches of PV within a study and to start with either neat or 1/10 pre-diluted serum, as neither of the variables significantly affected the IC_{50} results (Table 7).

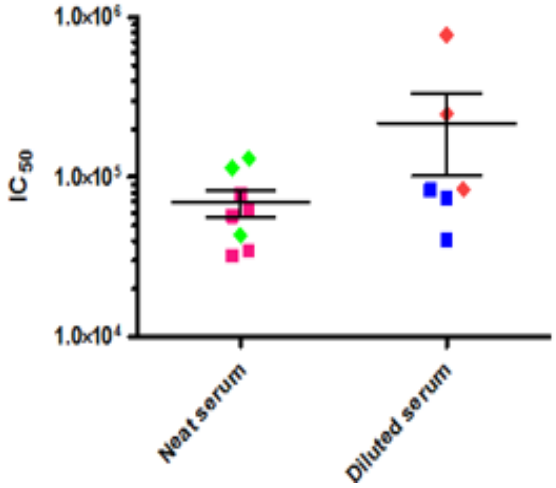


Figure 23. Comparison of IC_{50} values using different starting dilutions of serum; neat and diluted (pre-diluted 1/10).

In addition to looking at the difference in IC_{50} values, which is the most commonly reported output of a PVNA, the gradient of the neutralisation curves was analysed. The gradient of the neutralisation curve illustrates the trend in neutralisation of the PV, as the concentration of serum decreases. Both neat and pre-diluted equine sera were diluted two-fold across a 96-well plate and therefore theoretically the gradient of the curves should be the same. There is an expected shift along the independent x-variable axis due to a different starting dilution of serum (illustrated by Figure 24). Statistical analysis (using an unpaired T-test) confirmed that there was no significant difference between the gradients of the curves when comparing PV batch or serum dilution (Table 7), further highlighting the consistency of the PVNA.

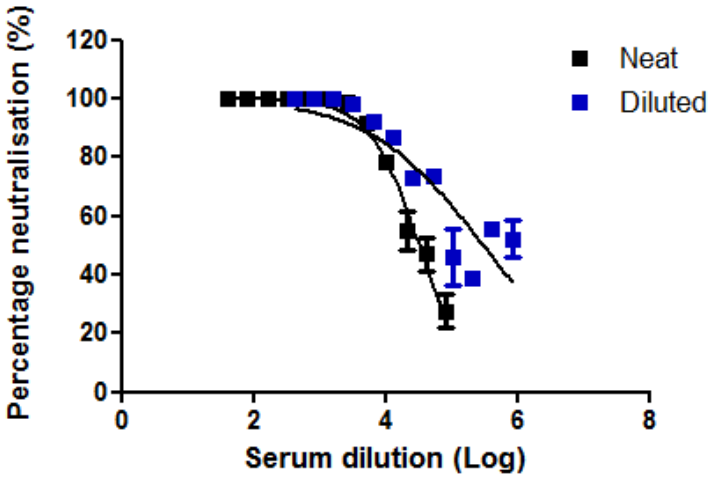


Figure 24. Comparison of neutralisation curves starting with either neat or pre-diluted serum. Mean and SEM of triplicate values are shown.

Table 7. Comparisons of IC_{50} values (orange) and gradients of neutralisation curves (grey), between different batches of PVs and starting dilution of serum (n = 3) using an unpaired T-test. Pre-diluted serum = starting dilution 1/400 and neat serum = 1/40.

	PV Batch 1 (pre-diluted)		**PV Batch 2 (neat)**	
PV Batch 1 (neat)	$p = 0.42$	$p = 0.53$	$p = 0.11$	$p = 0.79$
PV Batch 2 (pre-diluted)	$p = 0.22$	$p = 0.23$	$p = 0.26$	$p = 0.24$

4.3.2 Specificity of PVNA

To determine whether the PVNA can distinguish between homologous and heterologous antibodies, different combinations of PV and serum were assayed and mean IC_{50} values calculated (Table 9). Homologous pairings exhibited higher IC_{50} values than heterologous pairings except for the Nm/1/93 PV with sera raised in a ferret against Sx89 and SA/4/03. In both cases, the IC_{50} value was extrapolated from the inhibition curve because a serum dilution range at which there was 50% neutralisation was not reached. To test the accuracy of the extrapolated IC_{50} values, the two serum samples were diluted to the reciprocal of the IC_{50} previously obtained and tested in triplicate without titration (Table 8). Interestingly, these diluted sera did not bring about the expected 50% neutralisation and therefore it appears important to ensure that IC_{50} values are obtained empirically by diluting sera sufficiently to measure 50% neutralisation. In all cases where the IC_{50} value was extrapolated, the assays were repeated with further dilution of the serum and IC_{50} values recalculated. After diluting the Sx89 and SA/4/03 serum and assaying against the Nm/1/93 PV, IC_{50} values lower than the homologous Nm/1/93 titre were obtained (Table 9).

Table 8. PVs (A/equine/Newmarket/1979; Nm79 and A/equine/Sussex/1989; Sx89) were assayed against a single serum dilution, calculated as the reciprocal of the extrapolated IC_{50} value (to the nearest 10,000). Mean percentage neutralisation calculated (n=3).

WT PV	Serum (ferret)	Extrapolated IC_{50}		Serum dilution	% neutralisation
Nm79	Nm79	115175	»	120000	9.2
Sx89	Sx89	99239	»	100000	28.5

Table 9. Mean IC_{50} values of PVs against homologous and heterologous sera based on three replicates. **A**) post-infection ferret sera titrated to 1/51,200 with shaded boxes indicating where the IC_{50} value was obtained by extrapolation of the inhibition curve; **B**) post-infection sera titrated to 1/819,200; **C**) post-vaccination equine serum. Homologous pairings are underlined and heterologous pairings with higher IC_{50} values than homologous pairings are shown in red text.

A)	Antisera raised in ferrets				
Wildtype PV	**Nm/79**	**Sx/89**	**Nm/1/93**	**Nm/5/03**	**SA/4/03**
Nm/79	115175	1219	2951	18579	16809
Sx/89	1161	99239	4006	5187	2171
Nm/1/93	52786	819397	86748	54788	562464
Nm/5/03	83152	41544	74867	126096	33828
SA/4/03	41034	35922	20534	67902	107542

B)	Antisera raised in ferrets				
Wildtype PV	**Nm/79**	**Sx/89**	**Nm/1/93**	**Nm/5/03**	**SA/4/03**
Nm/79	**104294**	1238	3199	19077	17995
Sx/89	1610	**127532**	4934	5274	2501
Nm/1/93	58796	16134	**104950**	60597	40777
Nm/5/03	65349	43764	116223	**106163**	34448
SA/4/03	50869	40231	21364	41118	**152887**

C)	Antisera raised in equines		
Wildtype PV	**Nm/79**	**Sx/89**	**Rich/1/07**
Nm/79	24982	10270	12979
Sx/89	366	3437	1269
Rich/1/07	4512	26795	15916

4.3.3 Application of the PVNA for a vaccine efficacy trial

4.3.3.1 Virus neutralising antibody response

After completion of the vaccine efficacy trial, in which a recent FC2 EIV strain was used as the challenge virus for equines inoculated with two different clade specific vaccines (FC1 and FC2), all sera were tested by PVNA with a FC2 PV. Initially, all day 0 sera (n=27) were tested and compared to two known negative controls. All sera were negative using a cut-off of IC_{50} = 80 ($LogIC_{50}$ 1.9) as previously reported (Scott *et al.*, 2012). Furthermore, four negative control ponies were included in the vaccine efficacy trial and were bled at each of the five time points. All 16 samples pre-challenge had very low IC_{50} values although three were above the negative cut-off, with a mean IC_{50} of 158 ($LogIC_{50}$ 2.20). The positive threshold for this study was therefore set at an IC_{50} of 158 (or $LogIC_{50}$ 2.20).

PVNA measured seroconversion in the control ponies occurred as expected after experimental infection with the FC2 strain at 14 days post challenge (Figure 25E). The commercial vaccine group 6 seroconverted by day 28, with a peak on day 56 (Figure 25F), and the same was observed with groups 1 and 3 (Figures 25A&C); FC2 and FC1 high dose, respectively. The FC2 and FC1 low dose groups (2 and 4, respectively) showed partial seroconversion by day 28 but did not fully convert until day 56 (Figure 25B&D). Considering groups 1 to 4, irrespective of the vaccine strain or dose, overall the post challenge titres were significantly higher than pre-challenge (paired T-test; p=<0.001) and there was no significant difference between the post challenge responses (ANOVA; p = 0.081).

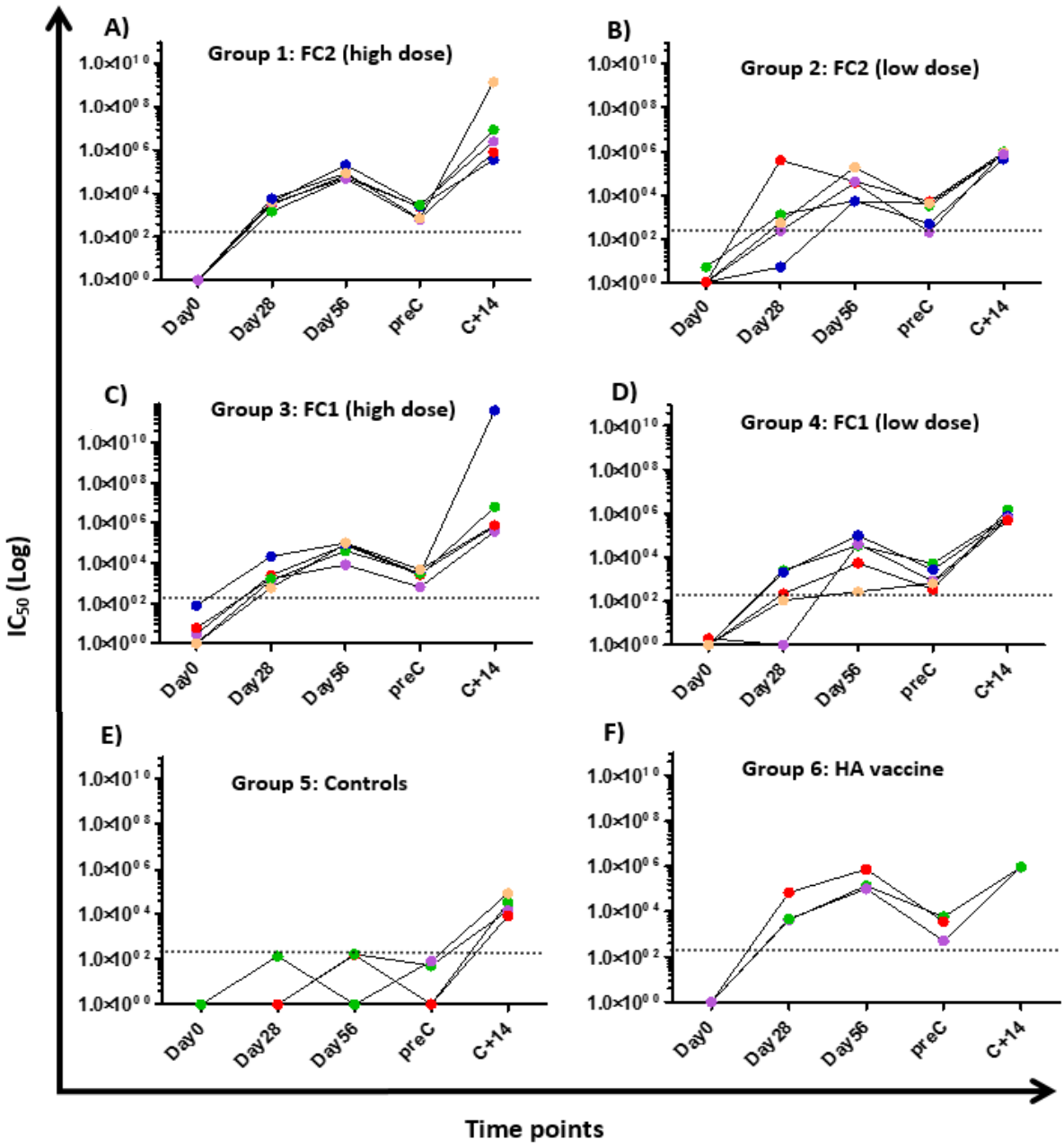


Figure 25. A-F. Neutralising antibody responses as measured by PVNA at five different time points during the vaccine efficacy trial; Day 0, Day 28, Day 56, pre-Challenge (preC) and 14 days post challenge (C+14). . Pony groups; **A)** FC2 (high dose), **B)** FC2 (low dose), **C)** FC1 (high dose), **D)** FC1 (low dose), **E)** controls and **F)** HA vaccine group. Antibody responses were recorded as IC_{50} values (reciprocal value of the serum dilution at which 50% neutralisation of the PV was reached). The positive response threshold is indicated by the dotted line at a $LogIC_{50}$ of 2.20. Coloured data points represent different ponies.

4.3.3.2 Comparison of SRH to PVNA responses

The correlation between antibody response to the FC2 antigen as measured by SRH and neutralising antibody response against the equivalent PV using PVNA was calculated between all samples (n=134) and demonstrated strong correlation (Figure 26; r = 0.84).

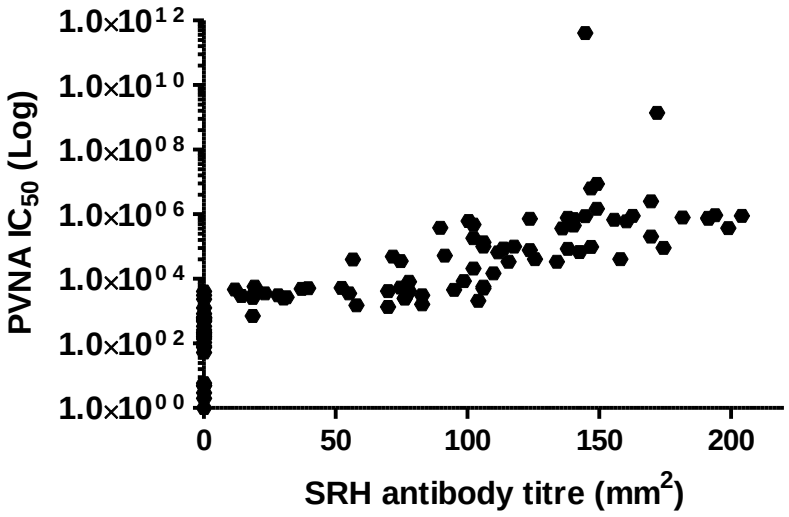


Figure 26. Correlation of antibody responses measured by pseudotype virus neutralisation assay and single radial haemolysis (n=134). Pearson's correlation coefficient r = 0.84, $p < 0.01$.

Notably the PVNA appears more sensitive than SRH at the pre-challenge time point; positive levels of neutralising antibodies were detected in the high-dose experimental vaccine and commercial vaccine groups (Figure 27A, C & F) whereas no haemolysis was visible to determine an SRH value from these serum samples (Figure 27B, D & E). Therefore, the PVNA appears to facilitate the measurement of remaining low antibody levels at a later time point after vaccination. At earlier stages of the immune response (Day 28) SRH values for individual ponies were more diverse, compared with the more homogenous results obtained with the PVNA.

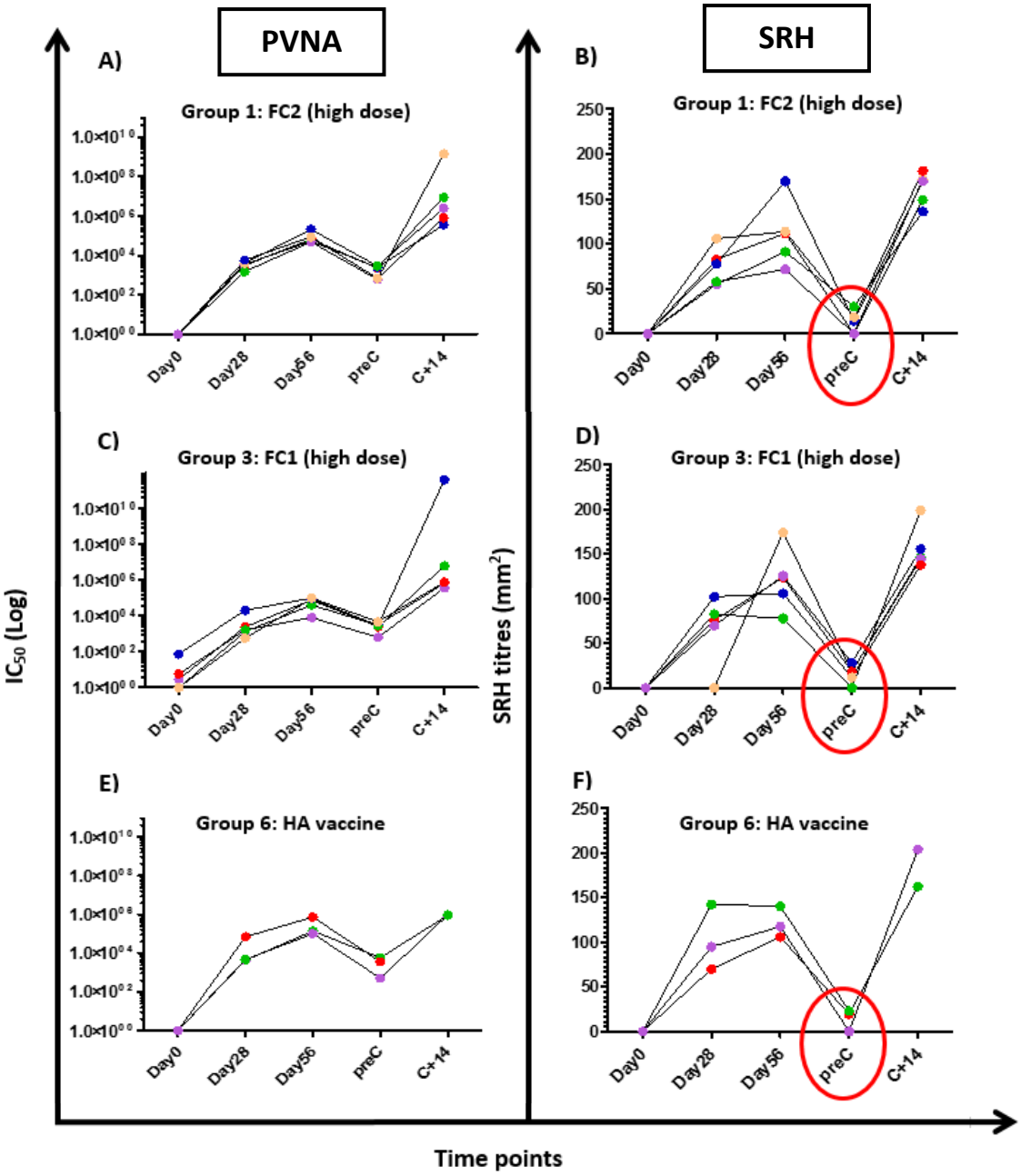


Figure 27. Neutralising antibody responses as measured by PVNA (**A**, **C**, **E**) compared to SRH (**B**, **D**, **F**). Responses were recorded at five different time points during the vaccine efficacy trial; Day 0, Day 28, Day 56, pre-Challenge (preC) and 14 days post challenge (C+14). Red circles highlight the point in time where SRH values are negative.

4.3.3.3 Comparison of HI to PVNA responses

The correlation between HI antibody response to the FC2 antigen and PVNA response against the equivalent PV was calculated between all samples (n=134) and demonstrated good correlation (Figure 28; r = 0.51).

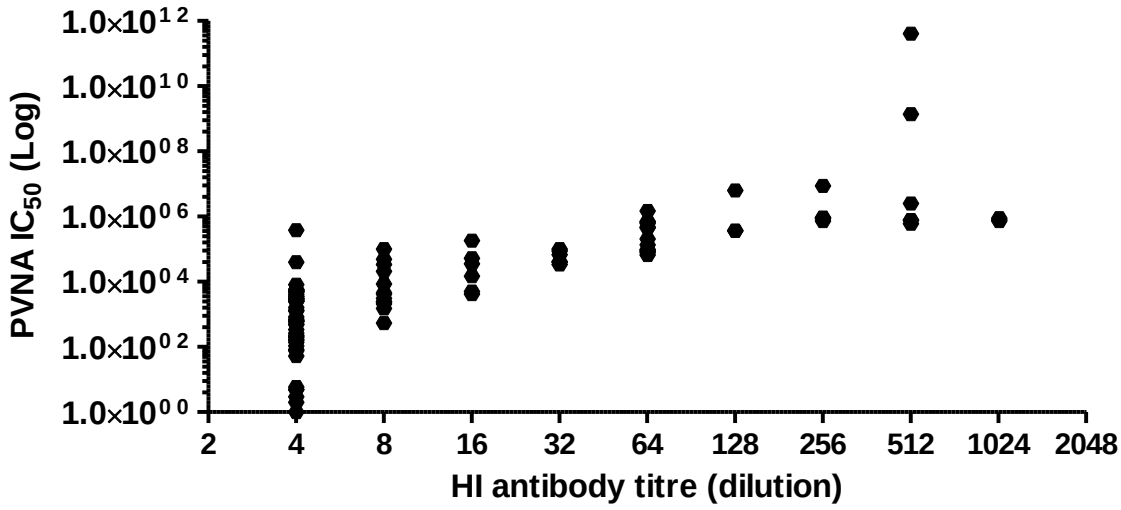


Figure 28. Correlation of antibody responses measured by pseudotype virus neutralisation assay and haemagglutinin inhibition (n=134). Pearson's correlation coefficient r = 0.51, $p < 0.01$.

Furthermore, and in agreement with the SRH results, the PVNA appeared to identify neutralising antibody responses in the cases where the HI antibody response was low (Day 28 and pre-challenge; Figure 29).

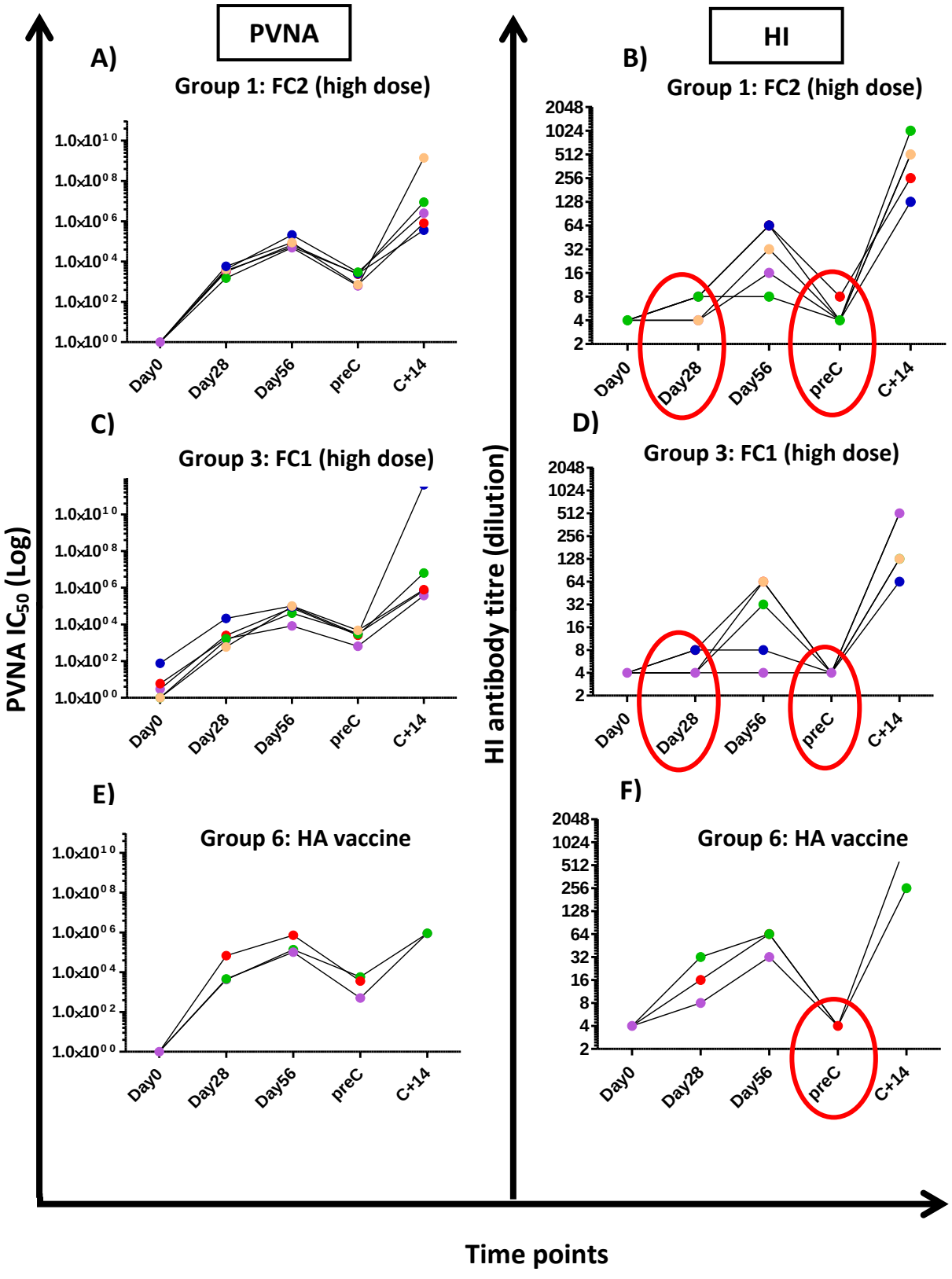


Figure 29. A-F. Neutralising antibody responses as measured by PVNA (IC_{50} values) (**A, C, E**) compared to HI titres (dilution) (**B, D, F**). Responses were recorded at five different time points during the vaccine efficacy trial; Day 0, Day 28, Day 56, pre-Challenge (preC) and 14 days post challenge (C+14). Red circled data points indicate negative results from HI.

4.4 Discussion

In order to assess the reproducibility of the equine influenza PVNA, a single batch of PV and a known positive, neat serum sample were assayed during a 24-hour period and then at minimum weekly intervals. At weekly intervals, the main variable was the passage number of the HEK293T/17 target cells. Based on statistical analysis, the results indicate that the assay can be conducted at different times without a significant change in IC_{50} readings. However, it should be taken into consideration that there was a log difference between the IC_{50} values in some assays and the reasons as to why this happened should be investigated further. Next, different batches of PVs and different initial dilutions of serum (neat or pre-diluted) were compared. Both variations did not have a detrimental effect on the consistency of results. This would be advantageous if limited volumes of sera are available.

In a prior study the PVNA was tested with equine influenza virus PVs and a threshold for negative samples (non-vaccinated Irish ponies) was set at <80, after direct comparison with serum samples negative by SRH (Scott *et al.*, 2012). This provided a starting point for determining positive and negative serum samples in the study but was adjusted to the mean value of the negative control ponies used in this study.

One difficulty that had to be overcome was choosing an appropriate dilution series. Monovalent serum samples against homologous viruses are likely to be highly neutralising, but knowing which serum dilution range to use in order to capture the point of 50% neutralisation is difficult. In cases where the dilution range was not broad enough, GraphPad Prism software extrapolated an IC_{50} value based on the dynamics of the curve, however this was not always accurate. It is not possible to accurately predict the IC_{50} if all serum dilutions cause more than 50% neutralisation of the PV. However, after pre-diluting the initial serum samples, 50% neutralisation was reached and an IC_{50} calculated. The exact method for calculating an average IC_{50} is often not reported. Results may or may not be transformed (Log10) before calculating the mean. Using the data set collected for this study, analysis was carried out using two methods; transforming the IC_{50} values, calculating the geometric mean and converting back to an IC_{50} value or simply calculating a geometric mean of all repeats without transformation. The difference in IC_{50} values was minimal, and always within the same two-fold serum

dilution range, indicating that either calculation method generates a very similar outcome.

In order to determine the specificity of the PVNA, a selection of sera and PVs were assayed in different combinations, both heterologous and homologous (Table 9). All homologous pairings led to higher levels of neutralisation for a given serum dilution and thus higher IC_{50} values than the heterologous pairings. For traditional assays such as HI, ferret serum is thought to be preferential for dissecting immune responses to equine influenza (Burrows & Denyer, 1982). Here, the PVNA was trialled with equine and ferret serum and both enabled heterologous and homologous pairings to be distinguished: Homologous pairings generated higher IC_{50} values.

Employing the PVNA in an equine influenza vaccine efficacy trial is the first known study of its kind. An assay that can measure neutralisation would be beneficial to deliver data on the neutralising antibody responses elicited post vaccination in comparison to the more traditional assays such as HI and SRH. HI measures the inhibition of agglutination between red blood cells and virus particles due to the presence of antibodies and SRH measures complement-mediated lysis of red blood cells, whereas the PVNA measures the neutralising antibody response. Comparative studies between the HI assay and the PVNA for human influenza have so far been carried out with promising positive results (Garcia & Lai, 2011; Yang *et al.*, 2014). Preliminary results using the PVNA for equine influenza were also promising with variable levels of neutralising antibody responses recorded, at different time points, post vaccination with two different monovalent vaccine strains. Post challenge there was a significant increase in neutralising antibody response for all immunised groups, indicative of seroconversion. Furthermore, despite some differences, there was strong correlation between the PVNA and SRH assay.

Differences in antibody responses measured by the different assays were observed, particularly at the pre-challenge stage, which may be explained by the previously reported increased sensitivity of the PVNA (Scott *et al.*, 2012). Antibody kinetics measured by the SRH assays are typically low around Day 28, post initial vaccination, and pre-challenge (Paillot *et al.*, 2010, 2015) but the PVNA indicated the presence of neutralising antibodies, particularly at the pre-challenge time point. As well as indicating an increased assay sensitivity it is possible that the neutralising antibody

response detected by the PVNA lasts longer than the SRH antibody response. The correlation between PVNA and HI was good but it is possible that the increased sensitivity in response of the PVNA at time points where HI responses were low, or negative, caused a decrease in the correlation coefficient.

Further analyses are warranted to define correlates of protection and strain specificity but confidentiality agreements with the Study Sponsor must be adhered to. It is important to consider that statistical significance does not necessarily infer a result to be biological meaningful i.e. a significant increase in post challenge titre does mean that the horse had clinical (signs of disease) and/or virological (detecting presence of viral RNA) protection.

5 IMPACT OF ANTIGENIC DRIFT ON NEUTRALISING ANTIBODY RESPONSES AGAINST EQUINE INFLUENZA HAEMAGGLUTININ

5.1 Introduction

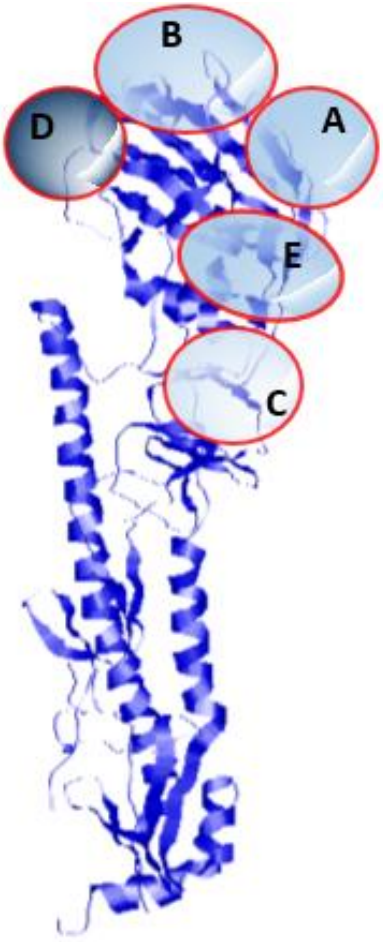
In 1979, a major equine influenza (EI) epidemic occurred across Europe (Burrows *et al.*, 1981; van Oirschot *et al.*, 1981). A vaccine was available at the time, which included the original equine influenza H3N8 subtype prototype strain A/equine/Miami/1963 (Miami/63), but it did not provide full protection, and vaccinated as well as unvaccinated horses were affected. Experimental infection with A/equine/Newmarket/1979 (Nm79) demonstrated that only 5% of ponies vaccinated against Miami/63 were protected against the new variant (Mumford *et al.*, 1983). Extensive outbreaks were also reported in Kentucky and California during 1980–81 and consequently, by 1982, vaccines against EI were recommended to include a new prototype strain; A/equine/Fontainebleau/1/79 or A/equine/Kentucky/1/81 (Hinshaw *et al.*, 1983). Reports of outbreaks in the UK declined until 1989 when notably, vaccinated horses were again affected. Multiple amino acid changes occurred within the HA1 sequence of the HA gene between 1979 (represented by Nm79) and 1989 (represented by A/equine/Sussex/89 - Sx89). Thus antigenic drift is thought to have reduced the efficacy of vaccines (Binns *et al.*, 1993), but the specific changes attributable to vaccine failure were not confirmed.

Three mutations were associated with changes in reactivity between virus and antibody in haemagglutination inhibition (HI) assays using mutant viruses generated with reverse genetics (Woodward *et al.*, 2015). The mutations in Sx89 compared to Nm79 occurred at amino acid residues 159 (aspartic acid, D was substituted for asparagine, N), 189 (asparagine, N was substituted for lysine, K) and 227 (proline, P was substituted for serine, S). Furthermore the mutations were compared to the putative EI HA antigenic epitopes, based upon the human influenza H3 (Daniels *et al.*, 1985) (Figures 30 & 31), and all three changes occurred within these putative epitope sites (B, B and A, respectively) (Figures 32 and 33).

```
          10         20         30         40         50
SQNPTSGNNT ATLCLGHHAV ANGTLVKTIT DDQIEVTNAT ELVQSTSIGK

          60         70         80         90         100
ICNNPYRVLD GRNCTLIDAM LGDPHCDVFQ YENWDLFIER SSAFSNCYPY

          110        120        130        140        150
DIPDYASLRS IVASSGTLEF TAEGFTWTGV TQNGRSGACR RGSADSFFSR

          160        170        180        190        200
LNWLTKSGDS YPTLNVTMPN NNNFDKLYIW GIHHPSTNNE QTKLYVQESG

          210        220        230        240        250
RVTVSTKRSQ QTIIPNIGSR PWVRGQPGRI SIYWTIVKPG DILMINSNGN

          260        270        280        290        300
LVAPRGYFKM RTGKSSIMRS DAPIDTCVSE CITPNGSIPN DKPFQNVNKV

          310        320
TYGKCPKYIK QNTLKLATGM RNVPEKQIR
```

Figure 30. A/equine/Newmarket/1979 HA1 (GenBank Accession number: KJ643908) annotated with the putative antigenic sites of H3 - **A**, **B**, **C**, **D**, and **E**.

Figure 31. Illustration of a haemagglutinin monomer (PDB: 2VIU) with putative antigenic sites circled in red (A-E). Protein figure edited using RasWin software (Version 2.7.5.2) and Microsoft PowerPoint.



```
                  10         20         30                  46
Nm79      SQNPTSGNNT ATLCLGHHAV ANGTLVKTIT DDQIEVTNAT ELVQSTSIGK
Sx89      SQNPTSGNNT ATLCLGHHAV ANGTLVKTIT DDQIEVTNAT ELVQSISIGK

              55
Nm79      ICNNPYRVLD GRNCTLIDAM LGDPHCDVFQ YENWDLFIER SSAFSNCYPY
Sx89      ICNNSYRVLD GRNCTLIDAM LGDPHCDVFQ YENWDLFIER SSAFSNCYPY

                                                 135  140
Nm79      DIPDYASLRS IVASSGTLEF TAEGFTWTGV TQNGRSGACR RGSADSFFSR
Sx89      DIPDYASLRS IVASSGTLEF TAEGFTWTGV TQNGTSGACK RGSADSFFSR

                159  163        171             187 189      196
Nm79      LNWLTKSGDS YPTLNVTMPN NNNFDKLYIW GIHHPSTNNE QTKLYVQESG
Sx89      LNWLTKSGNS YPILNVTMPN NKNFDKLYIW GIHHPSSNKE QTKLYIQESG

              207    213              227
Nm79      RVTVSTKRSQ QTIIPNIGSR PWVRGQPGRI SIYWTIVKPG DILMINSNGN
Sx89      RVTVSTERSQ QTVIPNIGSR PWVRGQSGRI SIYWTIVKPG DILMINSNGN

                260     267
Nm79      LVAPRGYFKM RTGKSSIMRS DAPIDTCVSE CITPNGSIPN DKPFQNVNKV
Sx89      LVAPRGYFKL RTGKSSVMRS DAPIDTCVSE CITPNGSIPN DKPFQNVNKV

                310
Nm79      TYGKCPKYIK QNTLKLATGM RNVPEKQIR
Sx89      TYGKCPKYIR QNTLKLATGM RNVPEKQIR
```

Figure 32. Alignment of A/equine/Newmarket/1979 (Nm79) (GenBank Accession number: KJ643908) and A/equine/Sussex/1989 (Sx89) (GenBank accession number: KJ643906) HA1 sequences. Sequence shown begins at amino acid after the 15-amino acid signal sequence. All amino acid changes between the two strains are highlighted. Red represents the three changes that form the focus of this study (positions **159, 189** & **227**) and yellow indicates all other amino acid substitutions. Affected antigenic sites A and B are highlighted in blue and green respectively.

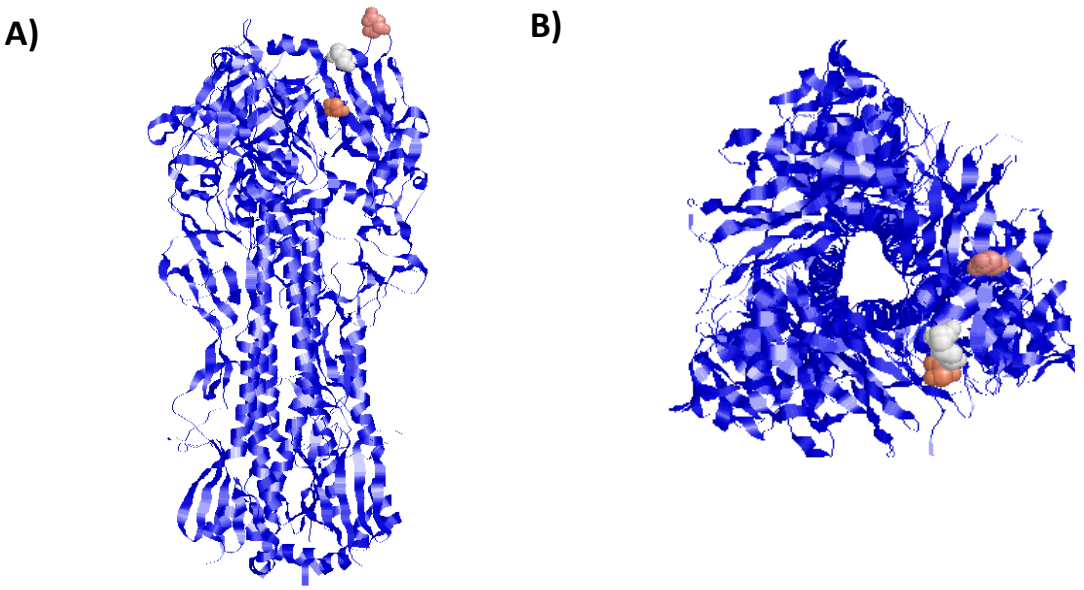


Figure 33. Trimeric HA structure of A/Newmarket/2/93 (PDB: 4UNW) highlighting amino acid residues at positions of 159 (pink), 189 (white) and 227 (orange), on one chain, from **A)** the side-view and **B)** top view of the protein. Protein figures edited using RasWin software (Version 2.7.5.2).

Newmarket is the home of horseracing in the UK and serious outbreaks of EI can lead to the cancellation of race meetings. Following the 1989 epidemic, Newmarket was again affected by EI in 1993 and 2003. Based on the geographical distribution of viruses, it was deduced that equine influenza viruses had diverged in the late 1980s into two lineages; American and Eurasian (Daly *et al.*, 1996). In 1993, Newmarket experienced outbreaks of EI from both American and Eurasian lineages, represented by A/equine/Newmarket/1/1993 (Nm/1/93) and A/equine/Newmarket/2/1993 (Nm/2/93) respectively. As a result, in 1995, it was recommended that EI vaccines be updated to include a representative of both lineages e.g. Nm/1/93 and Nm/2/93 (Anon, 1996). Moreover, in 2001, it was reported that the American lineage had further diverged into three sublineages; South American, Kentucky and Florida (Lai *et al.*, 2001). In 2003 an epidemic arose, represented by A/equine/Newmarket/5/2003 (Nm/5/03), and sequencing revealed that the virus was phylogenetically related to the Florida sublineage. This was the first isolation of its kind in the UK. Horses vaccinated against Nm/1/93 were not protected against the Nm/5/03 strain and showed clinical signs such as coughing and nasal discharge (Newton *et al.*, 2006). Furthermore, two unvaccinated horses were neurologically affected and had to be euthanased (Daly *et al.*, 2006). There were a total of eight amino acid substitutions between Nm/1/93 and Nm/5/03 within HA1 (Figure 34), three of which were situated within the antigenic epitopes identified for human influenza H3 HA at amino acid residues; 48 (isoleucine, I was substituted for methionine, M in antigenic site C), 190 (glutamine, Q was substituted for glutamic acid, E in antigenic site B) and 193 (glutamic acid, E was substituted for lysine, K in antigenic site B) (Figures 34 and 35).

```
                    5    10          20          30                   48
Nm/1/93       SQNPTSGNNT ATLCLGHHAV ANGTLVKTIT DDQIEVTNAT ELVQSISIGK
Nm/5/03       SQNPISGNNT ATLCLGHHAV ANGTLVKTIS DDQIEVTNAT ELVQSISMGK

                       58
Nm/1/93       ICNNSYRVLD GRNCTLIDAM LGDPHCDVFQ YENWDLFIER SSAFSNCYPY
Nm/5/03       ICNNSYRILD GRNCTLIDAM LGDPHCDVFQ YENWDLFIER SSAFSNCYPY

Nm/1/93       DIPDYASLRS IVASSGTLEF TAEGFTWTGV TQNGRSGACK RGSADSFFSR
Nm/5/03       DIPDYASLRS IVASSGTLEF TAEGFTWTGV TQNGRSGACK RGSADSFFSR

                                                      190 193
Nm/1/93       LNWLTKSGNS YPTLNVTMPN NKNFDKLYIW GIHHPSSNQQ QTELYIQESG
Nm/5/03       LNWLTKSGNS YPTLNVTMPN NKNFDKLYIW GIHHPSSNQE QTKLYIQESG

Nm/1/93       RVTVSTKRSQ QTIIPNIGSR PWVRGQSGRI SIYWTIVKPG DILMINSNGN
Nm/5/03       RVTVSTKRSQ QTIIPNIGSR PWVRGQSGRI SIYWTIVKPG DILMINSNGN

                                    272                 289
Nm/1/93       LVAPRGYFKL KTGKSSVMRS DAPIDICVSE CITPNGSIPN DKPFQNVNKV
Nm/5/03       LVAPRGYFKL KTGKSSVMRS DVPIDICVSE CITPNGSISN DKPFQNVNKV

Nm/1/93       TYGKCPKYIR QNTLKLATGM RNVPEKQIR
Nm/5/03       TYGKCPKYIR QNTLKLATGM RNVPEKQIR
```

Figure 34. Alignment of A/equine/Newmarket/1/1993 (Nm/1/93) (Partial sequence GenBank Accession number: CAA59415.3) and A/equine/Newmarket/5/2003 (Nm/5/03) (GenBank Accession number: ACI48804.1). All amino acid changes between the two strains are highlighted. Red represents the three changes that form the focus of this study (positions **48, 190** & **193**) and yellow indicates all other changes. Affected antigenic sites B and C are highlighted in green and purple respectively.

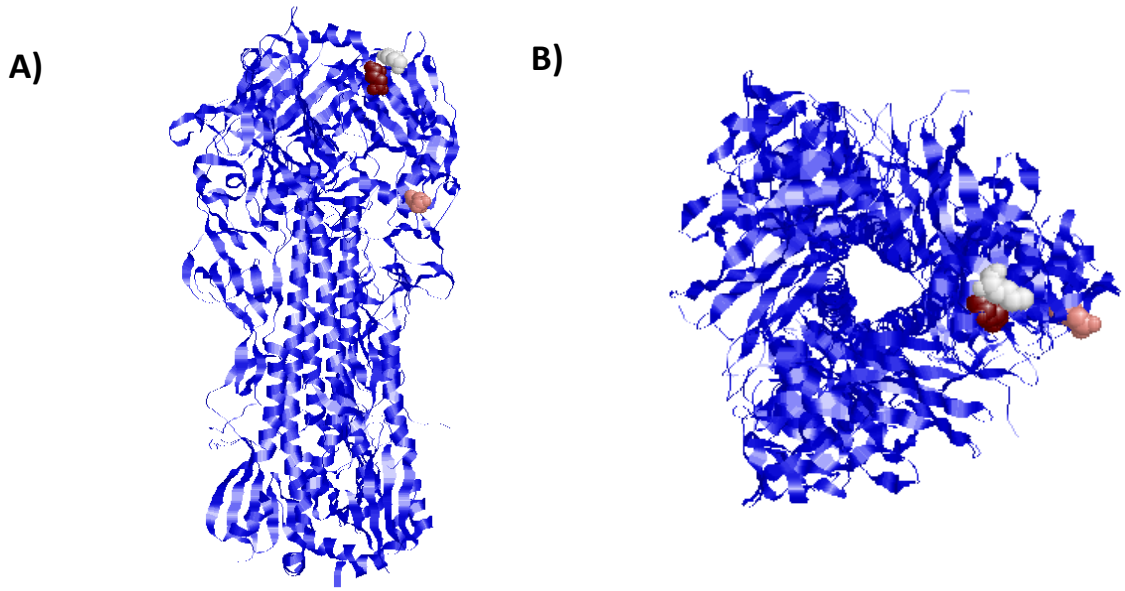


Figure 35. Trimeric HA structure of A/Newmarket/2/93 (PDB: 4UNW) highlighting amino acid residues at positions of 63 (pink), 190 (brown) and 193 (white), on one chain, from **A)** the side-view and **B)** top view of the protein. Protein figures edited using RasWin software (Version 2.7.5.2).

South Africa experienced its second ever outbreak of EIV in 2003, after the first severe outbreak in 1986 (Kawaoka & Webster, 1989). In both cases, it was believed to be a lack of biosafety measures that led to the outbreak in a naïve population of horses, not a case of vaccine breakdown (Guthrie, 2006; Guthrie *et al.*, 1999). However, when compared to the constituent vaccine strains at the time (Nm/1/93 and Nm/2/93), there were sufficient antigenic changes in the SA/4/03 virus to necessitate a vaccine update (OIE, 2006). Interestingly, sequence analysis of Nm/5/03 and A/equine/South Africa/4/2003 (SA/4/03) revealed that the viruses were related to two separate clades (clade 1 and 2 respectively) within the Florida lineage. The phylogenetic separation was distinguished by two amino acid differences at residues 78 (valine, V was substituted for alanine, A) and 159 (asparagine, N was substituted for serine, S) within HA1 (Figures 36 and 37). Both of these mutations were within putative antigenic sites (E and B, respectively).

```
                      10         20         30
Nm/5/03      SQNPISGNNT ATLCLGHHAV ANGTLVKTIS DDQIEVTNAT ELVQSISMGK
SA/4/03      SQNPISGNNT ATLCLGHHAV ANGTLVKTIS DDQIEVTNAT ELVQSISMGK

                                        78
Nm/5/03      ICNNSYRILD GRNCTLIDAM LGDPHCDVFQ YENWDLFIER SSAFSNCYPY
SA/4/03      ICNNSYRILD GRNCTLIDAM LGDPHCDAFQ YENWDLFIER SSAFSNCYPY

Nm/5/03      DIPDYASLRS IVASSGTLEF TAEGFTWTGV TQNGRSGACK RGSADSFFSR
SA/4/03      DIPDYASLRS IVASSGTLEF TAEGFTWTGV TQNGRSGACK RGSADSFFSR

                    159        170        180        190        200
Nm/5/03      LNWLTKSGNS YPTLNVTMPN NKNFDKLYIW GIHHPSSNQE QTKLYIQESG
SA/4/03      LNWLTKSGSS YPTLNVTMPN NKNFDKLYIW GIHHPSSNQE QTKLYIQESG

Nm/5/03      RVTVSTKRSQ QTIIPNIGSR PWVRGQSGRI SIYWTIVKPG DILMINSNGN
SA/4/03      RVTVSTKRSQ QTIIPNIGSR PWVRGQSGRI SIYWTIVKPG DILMINSNGN

Nm/5/03      LVAPRGYFKL KTGKSSVMRS DVPIDICVSE CITPNGSISN DKPFQNVNKV
SA/4/03      LVAPRGYFKL KTGKSSVMRS DVPIDICVSE CITPNGSISN DKPFQNVNKV

Nm/5/03      TYGKCPKYIR QNTLKLATGM RNVPEKQIR
SA/4/03      TYGKCPKYIR QNTLKLATGM RNVPEKQIR
```

Figure 36. Alignment of A/equine/Newmarket/5/2003 (Nm/5/03) (GenBank Accession number: ACI48804.1) and A/equine/South Africa/4/2003 (SA/4/03) (GenBank Accession number: ADB45165.1) highlighting the two amino acid changes between the two strains. Affected antigenic sites B and E are highlighted in light green and dark green respectively

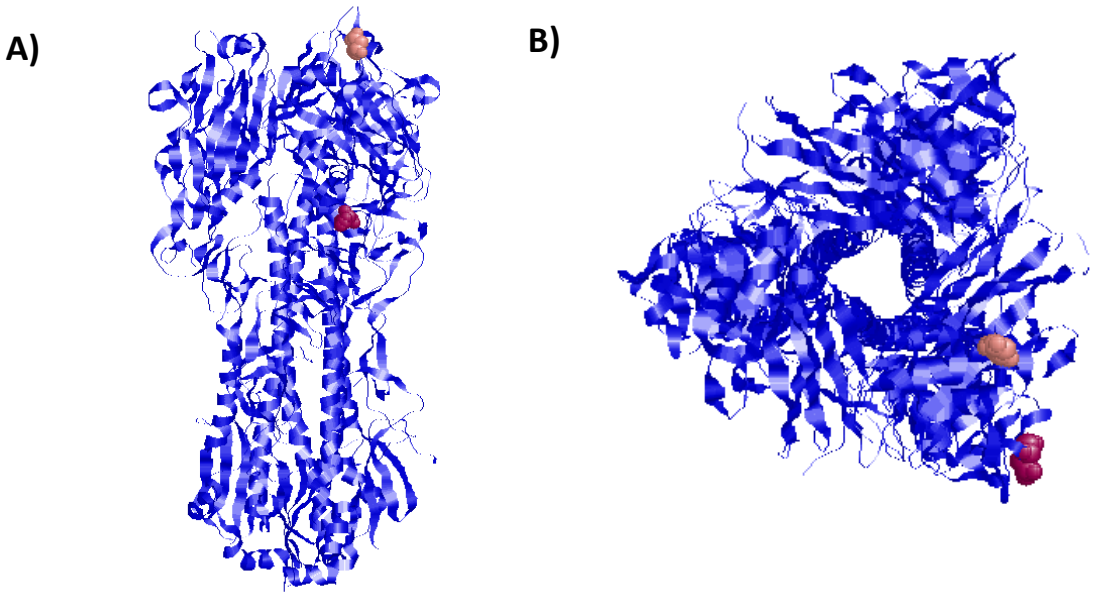


Figure 37. Trimeric HA structure of A/Newmarket/2/93 (PDB: 4UNW) highlighting amino acid residues at positions of 78 (pink) and 159 (brown), on one chain, from **A)** the side-view and **B)** top view of the protein. Protein figures edited using RasWin software (Version 2.7.5.2).

As mentioned earlier, studies have looked into the effect of amino acid changes using reverse genetics to generate equine (Woodward *et al.*, 2015) and human (Lin *et al.*, 2012) influenza mutant viruses, which can be used in HI assays. A method to map the results of HI assays with integrated HA sequence data was developed in 2004 and coined 'antigenic cartography' (Smith *et al.*, 2004). The method was initially employed for human influenza studies but has since been adopted for other species. Using this method, antigenic maps are generated as visual indicators of the antigenic viral clusters that evolve over time. An equine study highlighted how as few as two amino acid changes, within antigenically dominant regions, could result in virus escape from pre-existing immunity (Park *et al.*, 2009). Additionally, EI viruses from 1968 to 2007 were antigenically mapped and one amino acid change was found to be sufficient to determine a change between antigenic clusters. Notably, the changes were at amino acid residues 159 and 189 (Lewis *et al.*, 2011). Here, we employed a novel approach, using pseudotyped viruses to identify potentially important amino acid changes and determine how they affect neutralising antibody responses. Single amino acid mutations within the HA protein can be relatively easily introduced into a PV for use in a PVNA. This is achieved using site-directed mutagenesis to introduce the nucleotide changes into previously cloned HA genes, that code for the desired amino acid, within the HA

gene-plasmid construct before transfecting into producer cell lines. The PVs are a safer alternative to wild-type (WT) viruses mutated using reverse genetics and they also potentially allow dissection of antibody responses associated specifically with the HA glycoprotein.

The aim of this chapter was to look at the impact of single amino acid changes on H3 subtype specific neutralising antibody responses, using monospecific sera in pseudotype virus neutralisation assays (PVNA). Three outbreak scenarios were investigated:

(i) **Nm79** (D159N, N189K & P227S) and **Sx89** (N159D, K189N & S227P)
(ii) **Nm/1/93** (I48M, Q190E & E193K) and **Nm/5/03** (M48I, E190Q & K193E)
(iii) **Nm/5/03** (V78A & N159S) and **SA/4/03** (A78V & S159N)

5.2 Materials and methods

5.2.1 HA and NA plasmids

PCR products of WT Nm79 and Sx89 HA genes, as well as mutated HAs (Nm79; D159N, N189K & P227S. Sx89; N159D, K189N & S227P) were generously provided by Dr Adam Rash and Dr Debra Elton from the Animal Health Trust. Restriction enzymes *EcoR*V/*Xho*I and *BamH*I/*Xho*I were used to clone Nm79 and Sx89 PCR products respectively, via recognition sites incorporated into the PCR primers, into the mammalian expression vector pI.18. The WT Nm/1/93 HA sequence was kindly provided by Dr Janet Daly (as only partial sequence is available in NCBI database; GenBank Accession number: CAA59415.3). The gene was synthesised, cloned into pI.18 and subsequently mutated to generate the HA mutants (Nm/1/93; I48M, Q190E & E193K) by GenScript®. A PCR product of WT Nm/5/03 HA was also kindly provided by Dr Janet Daly. Site-directed mutagenesis (SDM) to produce the HA mutants (Nm/5/03; M48I, E190Q & K193E) and cloning into pI.18 using *BamH*I/*Xho*I restriction enzymes was carried out at the University of Kent. SA/4/03 and HA mutants were generated through SDM of Nm/5/03-pI.18 (Nm/5/03 - V78A & N159S; SA/4/03 - A78V & S159N). For detailed methods of cloning and PCR conditions for SDM see (Chapter 2 Methods section 2.1.9). Primer sequences for SDM are provided in Table 10.

Table 10. Primer sequences designed using Agilent Technologies QuikChange™ Site-Directed Mutagenesis Kit primer design program, for use in site-directed mutagenesis PCR.

Template sequence	Desired mutation	Primer sequence
Nm/5/03 WT	M 48 I	F 5’ gttgcatattttccctattgaaatgctctgaactaattctgtagc 3’
		R 5’ gctacagaattagttcagagcatttcaatagggaaaatatgcaac 3’
Nm/5/03 WT	E 190 Q	F 5’ aattttgtctgctgttgatttgagctcgggtgatgaatc 3’
		R 5’ gattcatcacccgagctcaaatcaacagcagacaaaatt 3’
Nm/5/03 WT	K 193 E	F 5’ gtcctgattcttggatgtacaactctgtctgctcttgatttgagctc 3’
		R 5’ gagctcaaatcaagagcagacagagttgtacatccaagaatcaggac 3’
Nm/5/03 WT	V 78 A	F 5’ aattctcatactgaaaggcgtcacagtgggggtc 3’
		R 5’ gctacagaattagttcagagcatttcaatagggaaaatatgcaac 3’
Nm/5/03 WT	N 159 S	F 5’ cacattcaatgtgggataagagcttccagattttgttagccaattc 3’
Nm/5/03 78A	N 159 S	R 5’ gaattggctaacaaaatctggaagctcttatcccacattgaatgtg 3’
Nm/5/03 190Q	K 193 E	F 5’ gtcctgattcttggatgtacaactctgtctgctgttgatttgagctc 3’
		R 5’ gagctcaaatcaacagcagacagagttgtacatccaagaatcaggac 3’
Nm/1/93 193K	Q 190 E	F 5’ attttgtctgctcttggtttgagctcgggtgatgaa 3’
		R 5’ ttcatcacccgagctcaaaccaagagcagacaaaat 3’
Nm/5/03 WT	E 190 Q & K 193 E	F 5’ gattcttggatgtacaactctgtctgctgttgatttgagctcgggtgatg 3’
		R 5’ catcacccgagctcaaatcaacagcagacagagttgtacatccaagaatc 3’

5.2.2 PV production and titration

PVs displaying only HA on their surface were produced as described in (Chapter 2 Methods section 2.3.1.1). Briefly, a four plasmid co-transfection of HEK293T/17 cells (using transfection reagent PEI) with plasmids expressing an equine influenza H3 surface glycoprotein, HIV gag-pol, firefly luciferase reporter gene and pCAGGS-TMPRSS2 endoprotease (kindly provided by Dr Eva Böttcher-Friebertshäuser) was employed. All PV titrations were performed as described previously and titres calculated in RLU/ml (Chapter 2 Methods section 2.3.2).

5.2.3 Serum samples and PVNA

Post infection ferret sera against Nm79, Sx89, Nm/1/93, Nm/5/03 and SA/4/03 as well as post vaccination equine sera against Nm79 and Sx89, containing subtype-specific antibodies, were all kindly provided by Dr Adam Rash and Dr Debra Elton at the Animal Health Trust for use in PVNAs. Neutralisation assays were performed as described in (Chapter 2 Methods section 2.3.3). Briefly, serum samples were serially diluted and mixed with PVs for 1hr at 37°C. HEK293T/17 cells were then added and the reagents incubated for 48hrs. Luciferase expression was quantified using BrightGlo reagent (Promega).

5.2.4 Statistical analysis

See Chapter 2 Methods section 2.3.4 for details of statistical analysis. Briefly, IC_{50} values were calculated using GraphPad Prism software and repeat values were averaged using a mean.

5.3 Results

5.3.1 Production of wild type and mutant equine HA PVs

All Nm79 PCR products were successfully cloned into pI.18 (WT shown in results section of Chapter 3, Figure 14). Cloning of Sx89 into pI.18 was also successful (Figure 38A & B).

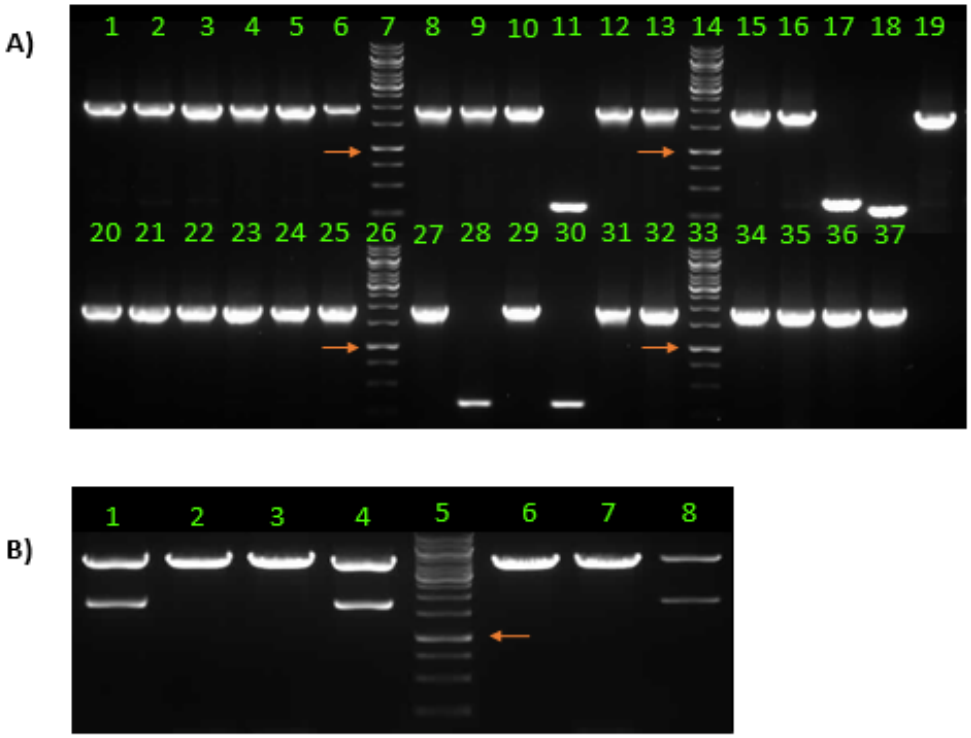


Figure 38. A) PCR screening of A/equine/Sussex/1/1989 (Sx89) WT, 159 and 189 colony lysates. Lanes 1-6, 8, 9, 12 and 13 were positive for Sx89 WT. Lanes 15, 16 and 19-23 were positive for Sx89 159 (17 and 18 were negative). Lanes 24, 25, 27, 31, 32 and 34-37 were positive for Sx89 189 (28 was negative). Lanes 10 and 29 were positive controls using pI.18-Nm79 WT. Lanes 11 and 30 were negative controls using pI.18 cut with *BamHI/XhoI*. **B)** Plasmid digest screen of Sx89 227 mutant; lanes 1 and 4 indicate positive clones. Lanes 7 and 8 were negative and positive controls (pI.18 cut with *BamHI/XhoI* and pI.18-Nm79 WT respectively). Orange arrows indicate 1Kb on DNA ladder.

Using site directed mutagenesis, all Nm/5/03 and SA/4/03 mutant HA-pI.18 plasmids were successful generated and verified by sequence analysis before use in transfections to produce PVs (Figure 39).

```
                          10         20         30
Nm/5/03 (WT)       SQNPISGNNT ATLCLGHHAV ANGTLVKTIS DDQIEVTNAT ELVQSISMGK
Nm/5/03 (V78A)     SQNPISGNNT ATLCLGHHAV ANGTLVKTIS DDQIEVTNAT ELVQSISMGK

                                                78
Nm/5/03 (WT)       ICNNSYRILD GRNCTLIDAM LGDPHCDVFQ YENWDLFIER SSAFSNCYPY
Nm/5/03 (V78A)     ICNNSYRILD GRNCTLIDAM LGDPHCDAFQ YENWDLFIER SSAFSNCYPY

Nm/5/03 (WT)       DIPDYASLRS IVASSGTLEF TAEGFTWTGV TQNGRSGACK RGSADSFFSR
Nm/5/03 (V78A)     DIPDYASLRS IVASSGTLEF TAEGFTWTGV TQNGRSGACK RGSADSFFSR

Nm/5/03 (WT)       LNWLTKSGNS YPTLNVTMPN NKNFDKLYIW GIHHPSSNQE QTKLYIQESG
Nm/5/03 (V78A)     LNWLTKSGNS YPTLNVTMPN NKNFDKLYIW GIHHPSSNQE QTKLYIQESG

Nm/5/03 (WT)       RVTVSTKRSQ QTIIPNIGSR PWVRGQSGRI SIYWTIVKPG DILMINSNGN
Nm/5/03 (V78A)     RVTVSTKRSQ QTIIPNIGSR PWVRGQSGRI SIYWTIVKPG DILMINSNGN

Nm/5/03 (WT)       LVAPRGYFKL KTGKSSVMRS DVPIDICVSE CITPNGSISN DKPFQNVNKV
Nm/5/03 (V78A)     LVAPRGYFKL KTGKSSVMRS DVPIDICVSE CITPNGSISN DKPFQNVNKV

                                                330
Nm/5/03 (WT)       TYGKCPKYIR QNTLKLATGM RNVPEKQIR
Nm/5/03 (V78A)     TYGKCPKYIR QNTLKLATGM RNVPEKQIR
```

Figure 39. Alignment of A/equine/Newmarket/5/2003 (Nm/5/03 WT) (GenBank Accession number: ACI48804.1) and Nm/5/03 V78A mutant HA sequences. Incorporation of amino acid change V78A (highlighted in red) was confirmed.

Titration assay results revealed successful production of WT and mutant Nm79, along with Sx89, Nm/1/93, Nm/5/03 and SA/4/03 PVs (Figure 40A, B and C). All PVs were compared to their respective delta envelope controls and all had significantly higher titres, indicating successful PV production ($p < 0.05$).

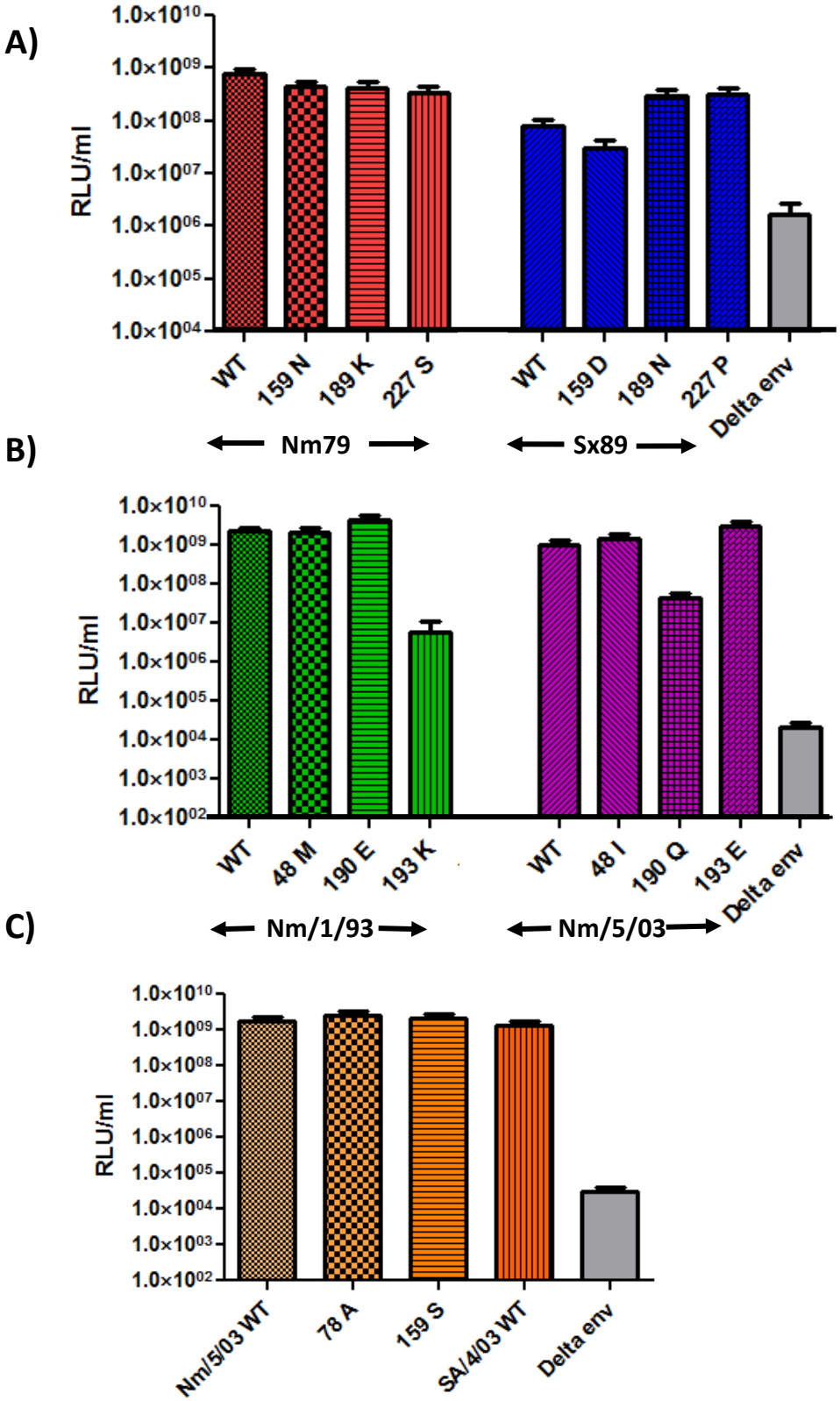


Figure 40. PV titres of **A)** A/equine/Newmarket/1979 (red) and A/equine/Sussex/1/1989 (blue). **B)** A/equine/Newmarket/1/1993 (green) and A/equine/Newmarket/5/2003 (purple). **C)** A/equine/Newmarket/5/2003 (brown) and A/equine/South Africa/4/2003 (orange). Wild type (WT) and HA mutant mean titres are expressed in relative luminescence units (RLU/ml) based on eight replicates +SEM. All PVs were significantly higher in titre than delta env ($p < 0.05$).

Interestingly the titres of Nm/1/93 193K and Nm/5/03 190Q, although functional, were significantly lower than the mean titres of the respective WT PVs (p<0.05). To understand if this was due to the mutation at specific residues, double mutants were produced. Initially, primers were designed to incorporate the 190E mutation into Nm/1/93 193K and for the introduction of 193E mutation into Nm/5/03 190Q. The primers designed to incorporate 190E into Nm/1/93 193K (Table 10) did not amplify the template DNA. Nm/5/03 was successfully amplified with primers designed to introduce 193E (Table 10), however sequence analysis confirmed that the mutagenesis was unsuccessful. New primers were designed to incorporate both 190Q and 193E into Nm/5/03, in the same PCR cycle (Table 10): Sequence analysis confirmed successful SDM. Furthermore, with both 190Q and 193E mutations present, the PV titre was significantly greater than with the single Nm/5/03 190Q mutant (p<0.01) (Figure 41).

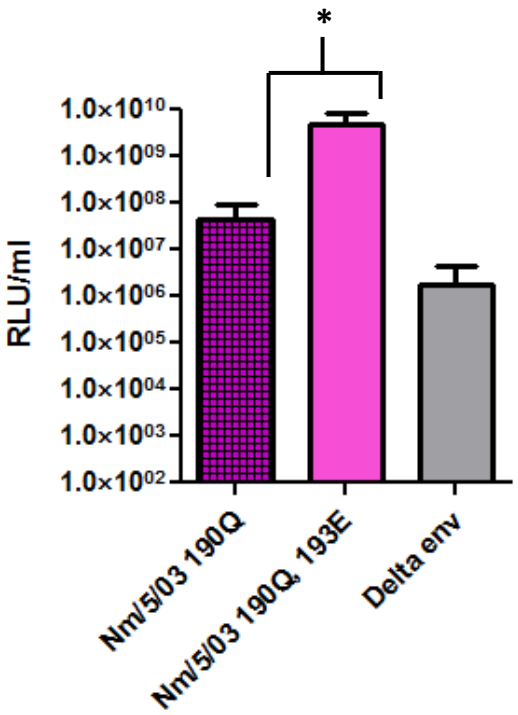


Figure 41. PV titres of A/equine/Newmarket/5/2003 (Nm/5/03) 190Q and Nm/5/03 190Q, 193E mutants. Mean titres are expressed in relative luminescence units (RLU/ml) based on eight replicates +SEM. * indicates a significant difference to Nm/5/03 190Q using an unpaired T-test for comparison (p<0.01).

5.3.2 Nm79 and Sx89 PVNAs

5.3.2.1 Ferret anti-sera

Nm79 and Sx89 PVs were initially used in PVNAs against their homologous serum pairings, which were raised in ferrets (F) and known to be positive for equine influenza H3 subtype-specific antibodies. Following this, fold-differences in IC_{50} values were calculated between each WT PV against its respective homologous and heterologous serum (Table 11). The IC_{50} for Nm79 WT PV with Sx89 serum was 60-fold less than the homologous pairing. Similarly, the IC_{50} for Sx89 WT PV with Nm79 serum was 50-fold less than its respective homologous pairing. Based on these findings it is evident that the PVNA can detect antigenic differences between viruses.

Table 11. IC_{50} results obtained from PVNAs using Nm79 and Sx89 WT PVs against respective homologous and heterologous ferret anti-sera. Fold-differences were calculated between the IC_{50} of the homologous and heterologous PV, for each serum sample. A negative fold-difference indicates a decrease in the efficacy of the serum to neutralise the PV. Mean IC_{50} values reported for each assay (n= minimum of 4). Anti-sera were raised in ferrets (F).

	Nm79 (F) serum		**Sx89 (F) serum**	
PV	**IC_{50}**	**Fold-difference**	**IC_{50}**	**Fold-difference**
Nm79 WT	**98793**	**1.00**	1238	-51.99
Sx89 WT	1610	-61.36	**64360**	**1.00**

Single mutant PVs were then compared by fold-difference to the WT PVs, when assayed against the same homologous serum sample. The homologous WT pairings yielded the highest IC_{50} values compared to the mutant viruses, with the exception of the 227 mutant, which was marginally higher (but less than 2-fold) than the WT for both Nm79 and Sx89 (Table 12). Interestingly, the Sx89/189N mutant reduced the IC_{50} by 16-fold. None of the other mutant viruses had greater than a 2-fold effect on the IC_{50}.

Table 12. IC_{50} results obtained from PVNAs with Nm79 and Sx89 mutant PVs and homologous serum (ferret, F). Fold-differences provide comparison of the WT PV to mutant viruses when assayed against the same serum. Mean IC_{50} values reported for each assay (n= minimum of 4).

	Nm79 (F) serum	
PV	**IC_{50}**	**Fold-difference**
Nm79 WT	**98793**	**1.00**
Nm79/159 N	73707	-1.34
Nm79/189 K	89603	-1.10
Nm79/227 S	116592	1.18
	Sx89 (F) serum	
Sx89 WT	**64360**	**1.00**
Sx89/159 D	33320	-1.93
Sx89/189 N	4021	-16.01
Sx89/227 P	68938	1.07

Furthermore, PVNAs were conducted with mutant PVs and heterologous sera. There was a decrease in IC_{50} with all Nm79 PVs assayed against Sx89 serum, compared to the IC_{50}s against homologous Nm79 serum. However, IC_{50} values obtained with both 159 and 189 mutants were more than 5-fold greater than with the WT, indicating that the introduction of these mutations (from the Sx89 strain) increased the PV neutralisation efficacy of the Sx89 serum (Table 13B).

The Sx89 WT PV tested with Nm79 serum mimics the serological scenario for a vaccinated horse at the time of the Sx89 outbreak: A 50-fold decrease in IC_{50} compared to the homologous serum pairing reflects the drop in immunogenicity. Interestingly the Nm79 serum displayed a 9-fold increase in ability to neutralise the Sx89/159D mutant (in direction of Nm79) compared to the Sx89 WT PV (Table 13A). The Sx89/189N and 227P mutants (again towards Nm79) showed less than a 2-fold change in IC_{50} with the Nm79 serum compared to the Sx89 WT PV.

Table 13. IC_{50} results obtained from PVNAs using ferret sera **A)** Nm79 PVs against heterologous Sx89 serum. **B)** Sx89 PVs against heterologous Nm79 serum. Sx89 WT + Nm79 serum mimics the outbreak scenario in nature (indicated in blue bold). Fold-differences provide comparison of the WT PV to mutant viruses when assayed against the same serum. Homologous serum and PV pairings are included in both tables for reference (highlighted in grey). Mean IC_{50} values reported for each assay (n= minimum of 4).

A)	Sx89 (F) serum	
PV	**IC_{50}**	**Fold-difference**
Sx89 WT	**64360**	**1.00**
Sx89/159 D	33320	-1.93
Sx89/189 N	4021	-16.01
Sx89/227 P	68938	1.07
	Nm79 (F) serum	
Sx89 WT	**1610**	**1.00**
Sx89/159 D	14716	9.14
Sx89/189 N	1804	1.12
Sx89/227 P	1033	-1.56

B)	Nm79 (F) serum	
PV	**IC_{50}**	**Fold-difference**
Nm79 WT	**98793**	**1.00**
Nm79/159 N	73707	-1.34
Nm79/189 K	89603	-1.10
Nm79/227 S	116592	1.18
	Sx89 (F) serum	
Nm79 WT	1238	1.00
Nm79/159 N	6253	5.05
Nm79/189 K	9148	7.39
Nm79/227 S	1280	1.03

5.3.2.2 Equine anti-sera

Following the PVNAs using ferret sera, sera raised against Nm79 and Sx89 viruses in horses were also trialled (E – equine). The IC_{50} for Nm79 WT PV against homologous Nm79 serum was almost 70-fold greater than the Sx89 WT PV against the Nm79 serum (Table 14), which highlighted the antigenic difference between the viruses. Contrary to expectations, the IC_{50} for Sx89 WT PV was 3-fold lower against the homologous Sx89 serum, compared to Nm79 WT PV (Table 14).

Table 14. IC_{50} results obtained from PVNAs using Nm79 and Sx89 WT PVs against respective homologous and heterologous equine (E) serum samples. Fold-differences were calculated between the IC_{50} of the homologous and heterologous PV, for each serum sample. A negative fold-difference indicates a decrease in the efficacy of the serum to neutralise the PV. Mean IC_{50} values reported for each assay (n= minimum of 3).

	Nm79 (E) serum		**Sx89 (E) serum**	
PV	**IC_{50}**	**Fold-difference**	**IC_{50}**	**Fold-difference**
Nm79 WT	**25482**	**1.00**	10270	2.99
Sx89 WT	366	-69.62	**3437**	**1.00**

When assayed against the homologous Nm79 serum, the Nm79 WT PV was most effectively neutralised and all Nm79 mutant PVs had lower IC_{50} values. The Nm79/189K mutant had the greatest impact on Nm79 serum, with a 6-fold decrease in IC_{50}. The Sx89 serum did not generate the highest IC_{50} against the Sx89 WT PV, although the differences to the mutants were marginal; mutants Sx89/159D and 227S increased the IC_{50} less than 2-fold and Sx89/189N decreased the ability of Sx89 serum to neutralise the virus, reducing the IC_{50} by 3-fold (Table 15).

Table 15. IC_{50} results obtained from PVNAs with Nm79 and Sx89 mutant PVs and homologous equine (E) serum. Fold-differences provide comparison of the WT PV to mutant viruses when assayed against the same serum. Mean IC_{50} values reported for each assay (n= minimum of 3).

	Nm79 (E) serum	
PV	**IC_{50}**	**Fold-difference**
Nm79 WT	**25482**	**1.00**
Nm79/159 N	16447	-1.55
Nm79/189 K	3978	-6.41
Nm79/227 S	16561	-1.54
	Sx89 (E) serum	
Sx89 WT	**3437**	**1.00**
Sx89/159 D	4485	1.30
Sx89/189 N	1271	-2.70
Sx89/227 P	4511	1.31

The equine serum samples were also tested against heterologous PVs. All of the Nm79 mutant PV IC_{50} values increased compared to Nm79 WT when tested against the Sx89 serum; Nm79/159N increased 1.5-fold, Nm79/189K 2-fold and Nm79/227S 3.5-fold (Table 16A). The Sx89 WT PV against Nm79 serum gave the lowest recorded IC_{50} at <400: A low IC_{50} was expected with this combination of PV and serum as it represents the outbreak scenario that occurred in nature in 1989. Results from Nm79 serum against Sx89/159D and Sx89/227P were incomplete. Interestingly, the Sx89/189N mutant increased the ability of Nm79 serum to neutralise the virus by 18-fold (Table 16B).

Table 16. IC_{50} results obtained from PVNAs with equine (E) serum **A)** Nm79 PVs against heterologous Sx89 serum. B**)** Sx89 PVs against heterologous Nm79 serum. Sx89 WT + Nm79 serum mimics the outbreak scenario in nature (indicated in blue bold). Fold-differences provide comparison of the WT PV to mutant viruses when assayed against the same serum. Homologous serum and PV pairings are included in both tables for reference (highlighted in grey). Mean IC_{50} values reported for each assay (n= minimum of 3).

A)

	Nm79 (E) serum	
PV	**IC_{50}**	**Fold-difference**
Nm79 WT	**25482**	**1.00**
Nm79/159 N	16447	-1.55
Nm79/189 K	3978	-6.41
Nm79/227 S	16561	-1.54
	Sx89 (E) serum	
Nm79 WT	10270	1.00
Nm79/159 N	14895	1.45
Nm79/189 K	22965	2.24
Nm79/227 S	35584	3.46

B)

	Sx89 (E) serum	
PV	**IC_{50}**	**Fold-difference**
Sx89 WT	**3437**	**1.00**
Sx89/159 D	4485	1.30
Sx89/189 N	1271	-2.70
Sx89/227 P	4511	1.31
	Nm79 (E) serum	
Sx89 WT	**366**	**1.00**
Sx89/159 D	*Data set incomplete*	
Sx89/189 N	6643	18.15
Sx89/227 P	*Data set incomplete*	

5.3.3 Nm/1/93 and Nm/5/03 PVNAs

Nm/1/93 and Nm/5/03 PVs were assayed against homologous and heterologous serum samples, raised in ferrets, in order to establish if the PVNA could identify the antigenic differences between them. Both homologous pairings of PV and serum gave the highest IC_{50} values. Heterologous pairings were both between 1 and 2-fold lower in IC_{50}. The difference between Nm/5/03 PV against Nm/1/93 serum and its homologous pairing was minimal at 1.16-fold decrease (Table 17).

Table 17. IC_{50} results obtained from PVNAs using Nm/1/93 and Nm/5/03 WT PVs against respective homologous and heterologous serum samples. Fold-differences were calculated between the IC_{50} of the homologous and heterologous PV, for each serum sample. A negative fold-difference indicates a decrease in the efficacy of the serum to neutralise the PV. Mean IC_{50} values reported for each assay (n= minimum of 3). Anti-sera were raised in ferrets (F).

	Nm/1/93 (F) serum		**Nm/5/03 (F) serum**	
PV	**IC_{50}**	**Fold-difference**	**IC_{50}**	**Fold-difference**
Nm/1/93 WT	**121942**	**1.00**	60597	-2.13
Nm/5/03 WT	105311	-1.16	**129222**	**1.00**

All mutant PVs tested against the respective homologous serum sample, had IC_{50} values within a 1.6-fold difference compared to the WT PV (Table 18). The result from Nm/5/03 190Q PV against Nm/5/03 serum was inconclusive.

Table 18. IC_{50} results obtained from PVNAs with Nm/1/93 and Nm/5/03 mutant PVs and homologous serum (ferret, F). Fold-differences provide comparison of the WT PV to mutant viruses when assayed against the same serum. Mean IC_{50} values reported for each assay (n= minimum of 2).

	Nm/1/93 (F) serum	
PV	**IC_{50}**	**Fold-difference**
Nm/1/93 WT	**121942**	**1.00**
Nm/1/93 48M	130988	1.07
Nm/1/93 190E	128578	1.05
Nm/1/93 193K	*Data set incomplete*	
	Nm/5/03 (F) serum	
Nm/5/03 WT	**129222**	**1.00**
Nm/5/03 48I	82991	-1.56
Nm/5/03 190Q	*Data set incomplete*	
Nm/5/03 193E	153504	1.19

Fold-differences between Nm/1/93 mutant PVs and Nm/5/03 serum were very similar to Nm/1/93 WT with less than a 1.5-fold increase in IC_{50} (Table 19A). Results for the Nm/1/93 193K mutant PV against Nm/5/03 serum and Nm/5/03 190Q against Nm/1/93 serum were not obtained due to the low titre PVs that could not be taken forward into the PVNA. The Nm/5/03 193E mutant increased the IC_{50} 3-fold, compared to the WT, when tested against Nm/1/93 serum (Table 19B).

Table 19. IC_{50} results obtained from PVNAs with **A)** Nm/1/93 PVs against heterologous Nm/5/03 serum. **B)** Nm/5/03 PVs against heterologous Nm/1/93 serum. Anti-sera were raised in ferrets (F). Fold-differences provide comparison of the WT PV to mutant viruses when assayed against the same serum. Homologous serum and PV pairings are included in both tables for reference (highlighted in grey). Mean IC_{50} values reported for each assay (n= minimum of 2).

A)

	Nm/1/93 (F) serum	
PV	**IC_{50}**	**Fold-difference**
Nm/1/93 WT	**121942**	**1.00**
Nm/1/93 48M	130988	1.07
Nm/1/93 190E	128578	1.05
Nm/1/93 193K	*Data set incomplete*	
	Nm/5/03 (F) serum	
Nm/1/93 WT	60597	1.00
Nm/1/93 48M	46643	1.30
Nm/1/93 190E	60992	1.01
Nm/1/93 193K	*Data set incomplete*	

B)

	Nm/5/03 (F) serum	
PV	**IC_{50}**	**Fold-difference**
Nm/5/03 WT	**129222**	**1.00**
Nm/5/03 48I	82991	-1.56
Nm/5/03 190Q	*Data set incomplete*	
Nm/5/03 193E	153504	1.19
	Nm/1/93 (F) serum	
Nm/5/03 WT	105311	1.00
Nm/5/03 48I	87375	-1.21
Nm/5/03 190Q	*Data set incomplete*	
Nm/5/03 193E	298713	2.84

5.3.4 Nm/5/03 and SA/4/03 PVNAs

As with the previous outbreak scenarios, homologous and heterologous serum samples were tested against Nm/5/03 and SA/4/03 PVs. In this scenario, with anti-sera raised in ferrets, the PVNA highlighted the antigenic difference between Nm/5/03 and SA/4/03 WT PVs when assayed against SA/4/03 serum with a 6-fold decrease (Table 20). However, there was a minimal difference between the two WT PVs when tested against Nm/5/03 serum (less than two fold).

Table 20. IC_{50} results obtained from PVNAs using Nm/5/03 and SA/4/03 PVs against respective homologous and heterologous serum pairings. Fold-differences were calculated on the difference between the homologous and heterologous pairings for each PV. A negative fold-difference indicates a decrease in the efficacy of the serum to neutralise the PV. Mean IC_{50} values reported for each assay (n= minimum of 2). Anti-sera were raised in ferrets (F).

	Nm/5/03 (F) serum		**SA/4/03 (F) serum**	
PV	**IC_{50}**	**Fold-difference**	**IC_{50}**	**Fold-difference**
Nm/5/03 WT	**75056**	**1.00**	34448	-5.87
SA/4/03 WT	102872	1.37	**202239**	**1.00**

Nm/5/03 78A and 159S mutants had less than a 2-fold increase in IC_{50} when tested against Nm/5/03 serum, compared to Nm/5/03 WT. The difference in neutralisation of the Nm/5/03 159S mutant against SA/4/03 serum was also minimal, with a 1.14-fold decrease in IC_{50}. The introduction of the 78A mutation (from SA/4/03) to Nm/5/03 decreased the IC_{50} 3-fold, which was the opposite of what might be expected.

Table 21. IC_{50} results obtained from PVNAs with Nm/5/03 and SA/4/03 mutant PVs and homologous serum (ferret, F). Fold-differences provide comparison of the WT PV to mutant viruses when assayed against the same serum. Mean IC_{50} values reported for each assay (n= minimum of 2).

	Nm/5/03 (F) serum	
PV	**IC_{50}**	**Fold-difference**
Nm/5/03 WT	**75056**	**1.00**
Nm/5/03 78 A	142476	1.90
Nm/5/03 159 S	70186	1.07
SA/4/03 WT	102872	1.37
	SA/4/03 (F) serum	
SA/4/03 WT	**202239**	**1.00**
Nm/5/03 78 A	74991	-2.70
Nm/5/03 159 S	178107	-1.14
Nm/5/03 WT	34448	-5.87

5.4 Discussion

The aim of this chapter was to generate PV mutants to look at the impact of single amino acid changes on equine H3 subtype specific neutralising antibody responses. Ferret and equine sera were used in PVNAs against the mutant PVs, to investigate the impact of the mutations that occurred during three different outbreak scenarios.

The first outbreak scenario had three amino acid changes between the Nm79 and Sx89 HA gene, which were highlighted by HI tests as potentially important antigenic differences (Woodward *et al.*, 2015). To confirm the ability of PVNAs to distinguish antigenically different viruses, WT PVs were trialled first. Anti-serum raised in ferrets against both Nm79 and Sx89 WT viruses exhibited higher IC_{50} values for homologous pairings than for heterologous, as expected.

The effect of each single mutant against the homologous serum pairing (raised in ferrets) was then investigated. Looking first at the Sx89 virus backbone with single Nm79 amino acid changes, the Sx89/189N mutant PV reduced the IC_{50} 16-fold against the Sx89 serum indicating that this particular mutation decreased the efficacy of the serum to neutralise the virus. This was more than seen for either of the 159D and 227P mutants: Sx89/159D mutant decreased the IC_{50} 2-fold and the IC_{50} against 227P did not change compared to WT Sx89. Mutating lysine to asparagine at position 189 in Sx89 does not introduce a potential N-linked glycosylation site but it is possible that the change interferes with the neutralising antibody response. Position 189 resides within the 190 helix of the HA, which forms part of the receptor binding site as well as antigenic site B (Woodward *et al.*, 2015). Interestingly, results using reverse genetics (RG) resulted in a similar finding, such that the highest decrease in HI titre was 100-fold with the 189N RG mutant and there was a 2.5-fold decrease with the 159D mutant. However, with the Sx89/227P RG mutant, despite no changes in neutralising antibody response being detected in the PVNA, a 22-fold titre decrease was observed using HI (Woodward *et al.*, 2015).

Considering the Nm79 virus backbone with Sx89 single amino acid changes, the mutation of asparagine to lysine at position 189 (Nm79/189K) did not reduce the neutralisation efficacy of the Nm79 serum, as was seen with the reciprocal Sx89/189N mutation. Similarly, Sx89/159D and 227P mutants had minimal effects on the IC_{50} with

less than a 0.5-fold decrease and increase, respectively. These results are in agreement with those obtained using mutants generated via reverse genetics; all three mutants had less than a 0.5 fold change in HI titre (Woodward *et al.*, 2015). It is possible that more than one single amino acid change is necessary to cause a decline in antibody recognition of epitopes (reflected in the IC_{50}). A triple mutant was generated using RG, with the Nm79 virus backbone, to incorporate the following mutations; T187S, N189K, V196I. Compared to the single N189K mutant, the triple mutant virus further decreased the HI titre 2-fold (Woodward *et al.*, 2015). In future work, it would be interesting to incorporate both 159 and 189 changes into Nm79 and Sx89 to observe the effect on the IC_{50}.

When testing the PV mutants against heterologous serum, an increase in IC_{50} was considered logical because the mutation increases the similarity of the PV towards the virus strain, to that which is homologous to the anti-serum. Nm79/159N and 189K increased the IC_{50} by 5 and 7-fold, respectively, whereas no difference was observed between the IC_{50} of Nm79/227S and Nm79 WT when assayed against Sx89 serum. Compared to the Sx89 WT PV, assayed against Nm79 serum, the Sx89/159D mutant increased the IC_{50} by 9-fold but less than a 0.6-fold difference was observed with Sx89/189N and 227P. In the PVNA, the change at position 159 effects the reactivity of both Nm79 and Sx89 with the heterologous serum but this does not hold true for HI tests (Woodward *et al.*, 2015). Amino acid changes at position 159 have been highlighted as a crucial change during the evolution of EI sublineages and a change of N159S currently distinguishes Florida clade 1 and clade 2 viruses (Bryant *et al.*, 2009). The HI test measures inhibition of virus binding due to the presence of antibodies but these do not necessarily correlate with neutralising antibody responses that are measured by the PVNA, which may explain the difference in results (reviewed in Kinsley et al., 2016). The mutation at residue 227, in either virus backbone, did not appear to greatly affect the IC_{50}. Neutralising antibodies are known to target the globular head of HA1 that encompasses amino acid positions 159 and 189 but not 227, therefore changes at position 227 may not have been detected by the PVNA (Figure 33).

Results from PVNAs with anti-serum raised in horses did not generate a trend as clear as that with ferret anti-sera. Unexpectedly, the heterologous pairing of Nm79 WT against Sx89 serum gave a 3-fold increase in IC_{50} compared to Sx89 homologous pairing.

Employing polyclonal serum samples can enable cross-reactive antibodies to increase an IC_{50} but in this case the serum was monoclonal; obtained from a horse vaccinated against Sx89 only. Equine serum is typically less specific than ferret serum, as discussed previously, and the serum samples available for this study differed in generation (post-vaccination and post-infection respectively). The homologous pairing of Nm79 WT PV and Nm79 serum gave a 70-fold increase in IC_{50} compared to Sx89 WT PV, as expected. It is interesting, however, that the Nm79 serum was polyclonal; the horse was vaccinated against Miami/63 followed by Nm79. A previous study showed minimal evidence of cross-reactivity between the Miami/63 virus and Sx89 serum with an HI titre of 4 (Daly *et al.*, 1996), which is likely to explain why the heterologous pairing of Sx89 and Nm79 serum was still considerably lower than the homologous pairing.

Following the WT PV assays against homologous and heterologous equine sera, the single-mutant PVs were trialled. The Nm79/189K mutant PV reduced the IC_{50} by 6-fold when assayed against Nm79 equine serum, which was not observed with the PV and ferret serum combination nor was it reported with mutant viruses in HI assays with anti-serum raised in ferrets (Woodward *et al.*, 2015). Compared to Nm79 WT against Nm79 serum, the IC_{50}s for Nm79/159N and Nm79/227S PV mutants differed by 0.5-fold, which agreed with the results obtained from ferret serum. A 3-fold decrease in IC_{50} was attributed to the Sx89/189N mutant against Sx89 serum, which although was not seen against ferret anti-serum with PVs, is in agreement with the RG mutant data (Woodward *et al.*, 2015). Therefore the impact of mutation at position 189 was highlighted in PVNAs using equine sera. The amino acid change in the Nm79 backbone from asparagine, a polar molecule, to lysine that carries a positive charge, may interfere with receptor binding and or antibody interactions. The impact of introducing asparagine into the Sx89 WT backbone was particularly prominent when assayed against the Nm79 equine serum with an 18-fold increase in IC_{50}. Therefore, the substitution of the charged lysine molecule increases the efficacy of Nm79 specific antibodies to neutralise the Sx89 virus.

The second outbreak scenario in the UK considered the antigenic differences between Nm/1/93 and Nm/5/03. Specifically, the only three amino acid changes that occurred within antigenic sites; sites A and B (Figure 34). Two of these changes were close together at positions 190 and 193, within antigenic site B and the 190 helix, which forms part of the receptor-binding site. Altering the amino acid combination at these

positions caused a decrease in PV titre for Nm/1/93 193K and Nm/5/03 190Q mutants. Due to the close proximity of the mutations it was possible that they were compensatory to one another. This theory was further supported by the increase in titre when Nm/5/03 190Q and 193E changes were introduced at the same time (Figure 41). Due to the low PV titres, PVNAs with Nm/1/93 193K and Nm/5/03 190Q mutant PVs were not completed. It would be beneficial to reduce the RLU input of these single mutants in future PVNAs and to trial the double mutant PV (Nm/5/03: 190Q, 193E). The only single mutation to affect IC_{50} by more than 2-fold was Nm/5/03 193E against Nm/1/93 serum. Interestingly, the change from lysine to glutamic acid does not alter the charged nature of the residues and therefore associated steric interactions were unlikely to be altered. At the time of the Nm/5/03 outbreak SRH antibody levels from vaccinated horses appeared adequate for protection and the differences in HA, to Nm/1/93, did not appear to constitute a cause for vaccine failure. These data are in agreement with the lack of fold-differences in IC_{50} values from the PVNAs. It was suggested that perhaps a combination of the antigenic changes and other pathogenicity factors resulted in the Nm/5/03 outbreak (Daly *et al.*, 2006). For example, a truncated NS1 gene was demonstrated to increase virulence of an equine influenza strain (Janet Daly pers. comm). There is also evidence to suggest that immunoreactivity may be found outside of the five epitopes (Kwaśnik *et al.*, 2015).

The final study investigated the difference between Nm/5/03 and SA/4/03. This outbreak scenario was attributed to a lack of biosafety measures in South Africa, not a case of vaccine breakdown (Guthrie *et al.*, 1999). As few as two amino acid changes distinguished the strains as two separate clades: Florida clade 1 (SA/4/03) and clade 2 (Nm/5/03), however, a vaccine update was deemed necessary. A study at the time showed that a Nm/1/93 vaccine boost would provide protection against SA/4/03 (Daly *et al.*, 2007b). Due to the slow process of updating equine influenza vaccines available on the veterinary market, such studies are valuable for determining what current vaccine(s) will provide a level of protection. The PVNA was employed to investigate the difference in neutralising antibody response between the two clades. There was a 6-fold decrease in IC_{50} between SA/4/03 and Nm/5/03 WT PVs against SA/4/03 serum, indicating a difference between the clades. However, the fold-difference between the WT PVs and Nm/5/03 serum was minimal at 1.4-fold. The Nm/5/03 159S mutant PV also

had a minimal effect on reactivity with either homologous or heterologous serum sample (1.07 and 1.14 fold-differences respectively). However, there was a 3-fold decrease in IC_{50} with the 78A mutant against SA/4/03 serum, which was the opposite to expectation but as mentioned earlier, whether or not this fold-difference is significant needs to be established.

Understanding the changes that cause equine influenza vaccine failure is complicated. Changes are continuously evaluated through improved serosurveillance programmes. Comparisons have been made to human H3 viruses, however, it is apparent that the same rules for cases of vaccine failure cannot be applied to equine influenza viruses. The rate of human H3 evolution is faster than equine H3: 0.87% amino acid changes over 14 years compared to 0.36% over 16 years (Skehel *et al.*, 1983). In human H3 strains, a minimum of four amino acid changes within two or more antigenic sites is attributed to drift variants with epidemiological implications (Wilson & Cox, 1990). However, for equine H3, eight amino acid changes that include changes within antigenic site B plus another site have led to major outbreaks (Woodward *et al.*, 2015). Following this work, mutant PVs may provide a tool to study the location of amino acids that affect neutralising antibody responses.

The difficulty in interpreting the PVNA results is to understand how a fold-change in IC_{50} relates to protection levels within the equine host. For HI, seroconversion is defined by a 4-fold increase or decrease in titre, but, at present, there is no such measure for the PVNA. Another point to address is that PVs with a titre less than $2x10^7$ RLU/ml is not routinely used in a standard PVNA protocol, equating to an RLU input of $1x10^6$ in 50µl. Therefore, either PV optimisation is necessary to increase titre or the RLU input for all PVs that are to be used in a comparative assay have to be reduced. The most important factor is that the RLU input is consistent across all PVs, if comparisons are to be made. Evidently, there will always be variation in the RLU and thus further work needs to be done to establish acceptable levels of variation for RLU input. Here, the Sx89/159 mutant PV had the largest RLU output range from $9x10^3$ – $6x10^5$ RLU. Whether this variation has led to inaccurate comparisons of neutralising antibody efficacy between PV mutants needs to be investigated further.

6 APPLICATION OF PSEUDOTYPED VIRUSES FOR SEROSURVEILLANCE AND POTENTIAL TO STUDY REASSORTMENT EVENTS

6.1 Introduction

Influenza is a classic zoonotic infection and the emergence of circulating subtypes in novel species is a long-standing global concern. The recent 2009 'swine' influenza pandemic serves as a potent reminder of the novel transmission events that can affect mankind (Isaacs, 2010; Smith *et al.*, 2009).

The H3N8 influenza virus subtype is one of the most abundant subtypes circulating in wild aquatic birds and it was also recently identified in domestic pigeons (Zou *et al.*, 2016). The virus is non-pathogenic in the intestine of aquatic birds, causing few or no clinical signs. Transmission events between different species of birds, birds to mammals, and mammals to mammals often occur as short-lived infections but the H3N8 virus, in particular, has become endemic in several mammalian hosts. The clinical signs associated with on-going outbreaks in horses, dogs and seals, tend to be worse than in avian hosts and in some cases are fatal (Anthony *et al.*, 2012; Crawford, 2005; Yondon *et al.*, 2013). Furthermore, the mammalian H3N8 host range extends to camels (Yondon *et al.*, 2014), donkeys (Qi *et al.*, 2010) and pigs (Tu *et al.*, 2009). Due to such a broad host range, the potential transmission to other mammalian species including humans is often considered. The H3 subtype is responsible for seasonal influenza outbreaks in humans and the potential for genetic reassortment of an H3 virus, leading to another pandemic, is a cause for concern (Kreibich *et al.*, 2013). For example, the Hong Kong pandemic of 1968 was caused by an H3 virus (Viboud *et al.*, 2005). Historically, reports indicated possible influenza transmission events from horses to humans over several centuries, illustrated by the large 1872 'epizooty' in the USA. However, the symptoms were general and could not be definitively attributed to influenza due to lack of available tests (Morens & Taubenberger, 2010). In 1965, five volunteers were infected with equine influenza; one individual suffered with febrile illness and the remaining four developed a high level of neutralising antibodies (Kasel *et al.*, 1965). Interestingly, the neutralising antibodies against the EI virus also elicited a response against a human strain of influenza. Then in 1967, EI was administered to a cohort of 33 volunteers and out of the 33, approximately two-thirds were infected but not all had symptoms or suffered with

illness (Alford *et al.*, 1967). Overall there is little evidence supporting human H3N8 infections after contact with infected horses (Burnell *et al.*, 2014). Although, a recent study identified seropositive horse handlers with multiple tests (microneutralisation assay, neuraminidase inhibition, enzyme-linked lectin assay and HI) against the A/equine/Ohio/2003(H3N8) virus strain in Iowa, U.S. (Larson *et al.*, 2015) and a review of papers dating back to 1959 also surmises that there is potential for human infection with equine influenza (Xie *et al.*, 2016).

A novel transmission event of the H3N8 virus from an equine to canine host was first reported in 2004 at a Greyhound racing facility in Florida, USA. Outbreaks across several states were then reported and it was subsequently declared endemic in the USA (Crawford, 2005). A separate transmission event of the H3N8 virus was also reported in foxhounds, in the United Kingdom (Daly *et al.*, 2008). Housing foxhounds within close proximity to horses demonstrated that it was possible for direct transmission of the virus to occur (Yamanaka *et al.*, 2009). However, prior to contracting the disease, the affected foxhounds had eaten an infected horse carcass including the lungs, which may provide an alternative explanation for transmission (Daly *et al.*, 2008). During the equine influenza outbreak in Australia 2007 interspecies transmission of the H3N8 virus to dogs was also recognised (Kirkland *et al.*, 2010).

Host sialic acid receptors are a known factor in determining transmissibility [reviewed in (de Graaf & Fouchier, 2014)]. Equine influenza viruses preferentially bind to α-2,3 linked sialic acid host receptors, which are present in the upper respiratory tract of horses (Suzuki *et al.*, 2000). A comparative study between horse and dog tracheal explants found α-2,3 receptors in the respiratory tract epithelium of dogs, providing a possible explanation for a direct transmission event (Daly *et al.*, 2008; Muranaka *et al.*, 2011). Phylogenetic analysis confirmed that the first reported outbreak strain (A/canine/Florida/43/04) was most closely related to isolates circulating in horses at the time and further comparisons, to other canine strains, identified two conserved amino acid changes at positions W222L and I328T in HA (Collins *et al.*, 2014; Crawford, 2005). These amino acid changes are believed to be important in facilitating the transmission of the equine H3N8 virus to the canine host; position 222 resides within the receptor binding site of HA1 and position 328 is the C-terminus of HA1 (Collins *et al.*, 2014).

Canine influenza has also independently appeared on another continent. The H3N2 virus was isolated in South Korea in 2007 (Song *et al.*, 2008) but a retrospective study highlighted that the virus was in fact circulating in China in 2006, based on a case with a pet dog in Guangdong, China (Li *et al.*, 2010). Within five years it was then isolated from roaming dogs in China (Su *et al.*, 2013). An undesirable environment are the dog farms in China, such close proximity of the dogs and mixing of individuals facilitates fast transmission of the virus and as an example, one farm showed 100% seropositivity as a consequence of the outbreak (Lee *et al.*, 2009). Similarly to the US H3N8 transmission event, direct transmission and the consumption of infected meat were thought to be the cause (Song *et al.*, 2008). However, sequence analysis confirmed that this virus was of avian origin. Avian influenza viruses share the same preferred binding specificity to α-2,3 linked sialic acid host receptors, which are found in the canine upper respiratory tract. Interestingly, the amino acid change at position 222 within the receptor binding site (tryptophan to leucine) found in the canine H3N8 virus was also present in the H3N2 virus.

China and South Korea are known for holding crowded animal markets, where birds and dogs are sold with the intention for consumption, which provides conditions for possible inter-species transmission. The transmission of canine influenza viruses, not only to other animals but to humans, provokes concern as dogs are widespread domestic companion animals. There have not been any reports of human infections with canine influenza virus thus far, but influenza isolates closely related to the human 1968 Hong Kong pandemic were previously isolated from dogs; H1N1, H2N2 and H3N2 (Chang *et al.*, 1976). Furthermore, H1N1 and H3N2 human isolates have recently been shown to replicate within canine tracheal explants (Gonzalez *et al.*, 2014).

Marine mammals are also known to be susceptible to type A influenza infections (Fereidouni *et al.*, 2014). For example, seals have been recently affected by multiple influenza A outbreaks with high mortality rates. New England, US (Anthony *et al.*, 2012) and Swedish coast lines (Zohari *et al.*, 2014) were two of the affected regions where hundreds of harbour seals (*Phoca vitulina*) died as a result of respiratory infection. An H3N8 virus was responsible for the outbreak in New England and an H10N7 subtype in Sweden. Phylogenetic sequence analysis indicated that both subtypes were of avian

origin. Studies have since investigated the receptor binding specificity of the seal H3N8 virus and found that it has an increased affinity for α2,6 linked sialic acid receptors, which are associated with human influenza virus infections. The virus infected human lung cells and could undergo respiratory transmission in a ferret model (Karlsson *et al.*, 2014). Although seals are less likely to come into contact with humans, compared with dogs and horses, the virus still poses a potential threat to human health. Haemagglutinin Inhibition (HI) assays were performed with pre and post human H3 seasonal vaccine serum samples, against the A/harbor seal/Massachusetts/1/2011 (H3N8) virus, and there was no evidence of cross-reactivity suggesting that the human population could be susceptible to the virus (Karlsson *et al.*, 2014).

The production of novel PVs, such as canine and phocine (seal) influenza pseudotypes, would provide a new platform for sero-surveillance of these viruses particularly in the case of seals as it is problematic to take samples from wild animals during acute infection. Moreover, developing tools to understand the factors that enable successful transmission events is of primary interest to public health organisations. We have so far demonstrated that it is possible to produce an equine H3N8 pseudotyped virus. Further to this, the production of PVs with novel HA/NA strain combinations, may provide an insight into viable HA/NA pairings from a transmission prediction perspective. We have also demonstrated that it is possible to produce mutant HA PVs, which could be valuable tools to study the effect of mutations on receptor binding specificity and virus entry. An advantage of using non-replicative PVs for this work is the lowered risk of working with novel (potentially harmful) combinations of HA/NA.

The objectives of the work described in this chapter were to; i) generate canine and phocine influenza virus pseudotypes; ii) employ the phocine PVs in PVNAs to detect neutralising antibodies in wild seal serum samples and/or vaccinated control samples, respectively; iii) introduce single canine influenza HA mutations into Nm/5/03 equine H3 PV for use in red blood cell binding assays and iv) investigate the generation of novel subtype combinations of HA/NA PVs. The overall aim was to generate pseudotype viruses as tools for serosurveillance and their potential application for studying reassortment events.

6.2 Materials and methods

6.2.1 HA, NA and protease plasmids

Canine HA genes; H3 A/canine/Colorado/30604/2006 (H3N8) (GenBank accession number: AB537183.1) and H3 A/canine/Guangdong/3/2011 (H3N2) (GenBank accession number: JX195358.1) were synthesised, incorporating *BamHI*/*KpnI* and *EcoI*/*BglII* restriction sites, respectively, and cloned into pUC57 by Genscript® (Central, Hong Kong). Genscript® also provided both canine HA genes cloned in mammalian expression vector pDREAM2.1.

The seal H3 A/harbor seal/Massachusetts/1/2011 (H3N8) (GenBank accession number: JQ433879.1) gene was synthesised, incorporating *KpnI*/*SalI* restriction sites and cloned into pUC57 by Bio Basic Inc® (Ontario, Canada). Upon receipt, the cloned DNAs were resuspended, re-transformed and HA genes subcloned into pI.18 (Chapter 2 Methods sections 2.1.2 and 2.1.3). The A/chicken/Germany/N49 H10 HA clone was kindly provided by Dr Davide Corti (Institute for Research in Biomedicine, Bellinzona, Switzerland).

Site directed mutagenesis of the A/equine/Newmarket/5/2003 (H3N8) HA gene was performed as described in (Chapter 2 Methods section 2.1.9).

NA genes 1, 2, 4, 5 and 7 (Table 22) cloned in pI.18 were all kindly provided by Dr Nigel Temperton (Viral Pseudotype Unit, Kent) and re-transformed from original stocks. NA genes 3 & 6 (Table 22) were synthesised, incorporating *EcoRI/XhoI* and *BamHI*/*XhoI* restriction sites respectively and cloned into pUC57 by GenScript®. Upon receipt, the cloned DNA were resuspended, re-transformed and NA genes subcloned into pI.18 (Chapter 2 Methods sections 2.1.2 and 2.1.3).

Table 22. Influenza virus strains from which neuraminidase genes were isolated for pseudotype studies, including full subtype and associated GenBank accession numbers.

	Neuraminidase		GenBank accession number
	Isolate	**Subtype**	
1	A/chicken/Italy/1067/1999	H7**N1**	CAC95055.1
2	A/Udorn/307/1972	H3**N2**	DQ508931
3	A/canine/Guangdong/3/2011	H3**N2**	JX195360.1
4	A/chicken/Pakistan/34669/1995	H7**N3**	AAO62018.1
5	A/turkey/Ontario/6118/1968	H8**N4**	EU429793.1
6	A/equine/Richmond/1/2007	H3**N8**	KF559336.1
7	A/avian/Shanghai/2/2013	H7**N9**	AGL44440.1

Plasmids expressing the pCAGGS-TMPRSS2 and pCAGGS-HAT endoproteases were kindly provided by Dr Eva Böttcher-Friebertshäuser and Professor Hans Dieter Klenk (Institute of Virology, Philipps University Marburg, Germany). Other protease expression plasmids; pcDNA3.1-TMPRSS3, pCMV-Tag3-TMPRSS4 and pcDNA3.1-TMPRSS6 were a kind gift of Professor Stefan Pöhlmann (Infection Biology Unit, German Primate Center, Germany). The Kallikrein 5 (KLK5) encoding plasmid was originally purchased from Genscript® and kindly provided by Dr Nigel Temperton (Viral Pseudotype Unit, Kent).

6.2.2 PV production and titration

PVs displaying solely HA on their envelope were produced as described in (Chapter 2 Methods section 2.3.1.1). Briefly, a four plasmid co-transfection of HEK293T/17 cells (using transfection reagent PEI) with plasmids expressing an influenza HA surface glycoprotein, HIV gag-pol, firefly luciferase reporter gene and endoprotease. For production of HA and NA PVs, a five plasmid co-transfection protocol was used with the addition of 125ng of NA plasmid (4:1 ratio of HA:NA, in an attempt to mimic ratio on wild type virus). All PV titrations were performed as described previously and titres calculated in RLU/ml (Chapter 2 Methods section 2.3.2).

6.2.3 Serum, ELISA and PVNA

Wild seal sera for use in PVNAs were obtained from seals caught in the Baltic Sea (North-western Europe; n=5) and Caspian Sea (Europe/Asia border; n=11), which were kindly provided by Sasan Fereidouni (University of Veterinary Medicine, Vienna). Sera from seals inhabiting the Pacific Ocean (n=30) were gifted from Frances Gulland (The Marine Mammal Center, California). All sera were tested by competitive ELISA for influenza nucleoprotein (not subtype specific), using a commercial kit developed at Friedrich-Loeffler-Institut (ID Screen, ID.VET).

Control serum; positive H3 serum was from a horse that had been vaccinated with several H3 equine influenza strains (Scott *et al.*, 2012), positive H10 serum was from a vaccinated chicken obtained from the World Organisation for Animal Health (OIE) and positive influenza B serum (anti-B/Brisbane/60/2008) was from NIBSC (11/136 sheep). Fetal bovine serum (FBS) was used as a negative control.

PVNAs were performed as described in (Chapter 2 Methods section 2.3.3). Briefly, serum samples were serially diluted and mixed with PV supernatent for 1hr at 37°C. HEK293T/17 cells were then added and the reagents incubated for 48hrs. Luciferase expression was quantified using BrightGlo reagent (Promega).

6.2.4 Neuraminidase activity assays

Influenza MUNANA neuraminidase activity assays were used to assess the neuraminidase activity associated with PVs displaying both HA and NA glycoproteins (Chapter 2 Methods section 2.4).

6.2.5 Statistical analysis

Comparison of two titration assay data sets was carried out using an unpaired T-test, except in cases where a normal distribution could not be assumed. In such cases, the Mann-Whitney test was applied (indicated when applied in results section). Values outside of the 95% confidence limits were deemed to be statistically significant. GraphPad Prism version 5 software was used for analysis.

6.3 Results

6.3.1 Production of canine influenza PVs

Subcloning of the HA genes from the H3N8 A/canine/Colorado/30604/2006 (Colo06) and H3N2 A/canine/Guangdong/3/2011 (Guang11) into plasmid vector pI.18 was successful, using restriction enzymes *BamHI*/*KpnI* and *EcoI*/*BglII*, respectively. Positive clones were confirmed by colony PCR. Following this, the standard transfection protocol (Chapter 2 Methods section 2.3.1) was employed in an attempt to produce H3-only PVs. In addition to the pI.18 HA-encoding plasmids, two further transfections were carried-out simultaneously, using an alternative mammalian expression vector, pDream, harbouring the Colo06 and Guang11 HA genes. By comparison to the delta env negative control, pI.18-Colo06 facilitated the production of a functional PV (p = 0.007) however, pDream-Colo06 did not (p = 0.182). Neither pI.18 nor pDream expression vectors encoding the Guang11 HA, generated a functional PV (p = 0.186 and 0.184 respectively) (Figure 42).

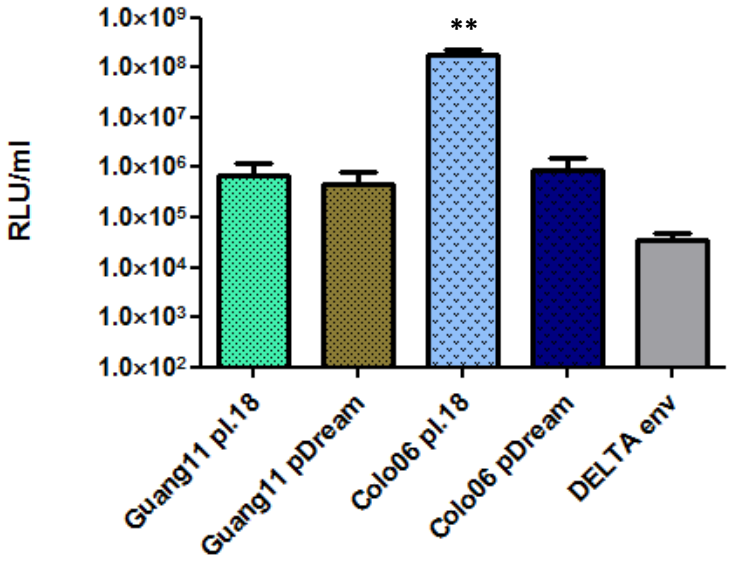


Figure 42. Attempted generation of H3-only PVs derived from A/canine/Colorado/30604/2006 H3N8 (Colo06) and A/canine/Guangdong/3/2011 H3N2 (Guang11) strains using different HA-encoding plasmid vectors; pDream and pI.18. Mean PV titres are reported in relative luminescent units (RLU/ml) based on eight replicates with error bars indicating +SEM. ** Indicates a significant difference (p < 0.05) determined by an unpaired T-test.

Troubleshooting the lack of Guang11 H3 PV generation began with protease optimisation. Transfections with different quantities of TMPRSS2 and HAT proteases, which have facilitated the generation of other H3 PVs previously, were trialled (125-500ng) but all yielded titres not significantly different to that of the delta env control. Other available proteases were subsequently trialled; TMPRSS3, TMPRSS4, TMPRSS6 and Kallikrein 5 (KLK5) but again, none were successful in generating PVs (Figure 43).

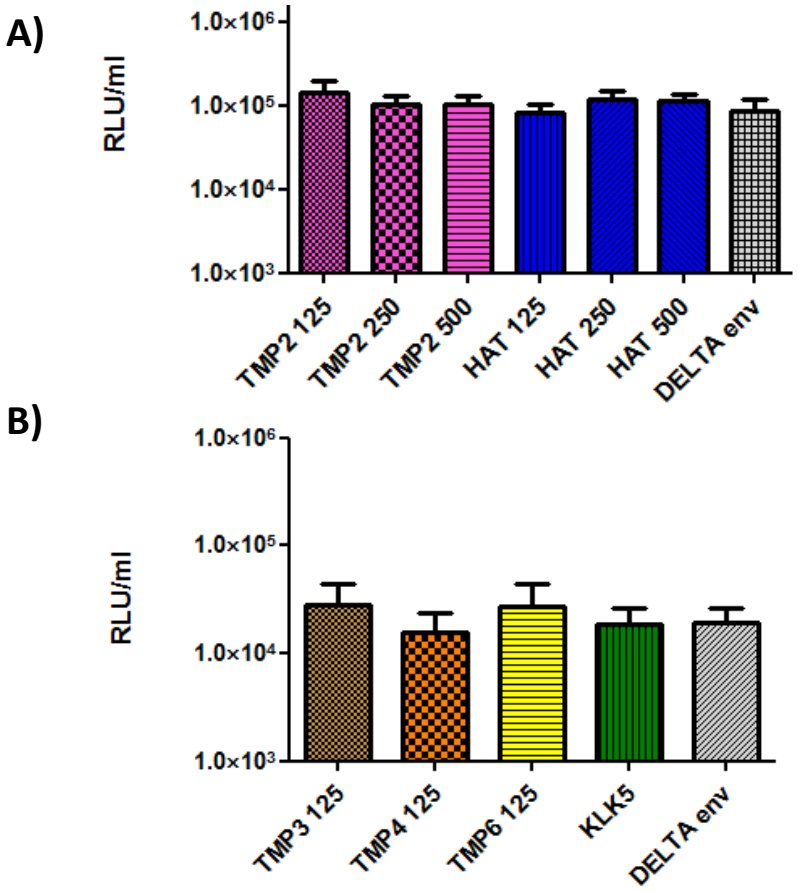


Figure 43. Testing A/canine/Guangdong/3/2011 H3-only PV generation with **A)** TMPRSS2 (TMP2) and HAT. **B)** TMPRSS3 (TMP3), 4 (TMP4), 6 (TMP6) and Kallikrein 5 (KLK5) proteases. Number indicates the quantity of protease expressing plasmid used (ng), if not stated 125ng was used. Mean PV titres are reported in relative luminescent units (RLU/ml) based on eight replicates with error bars indicating +SEM.

The sequence at the HA cleavage site of Guang11 H3 was compared to that of other PVs that have been successfully generated. Whilst Guang11 is different to equine H3s, its cleavage site is the same as a strain of seal influenza H3, which was successfully pseudotyped using both TMPRSS2 and HAT (Figure 44). It is therefore likely to be another aspect of PV production that is preventing generation of this particular strain, not the cleavage site/choice of protease.

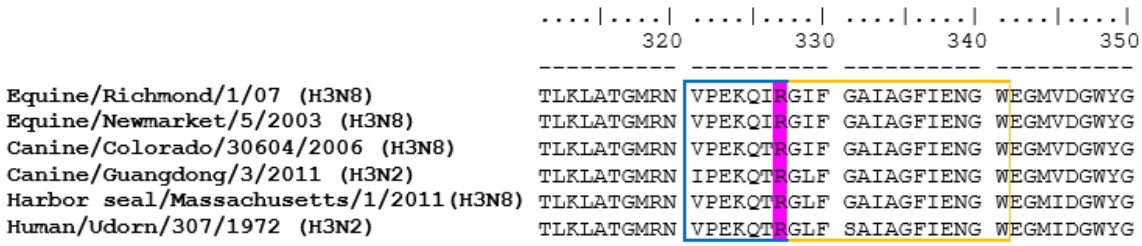


Figure 44. Alignment of H3 cleavage sites from different species; equine, canine, human and seal H3. Blue box indicates protease cleavage region with the arginine (R) cleavage site highlighted in pink. Yellow box indicates fusion peptide.

Our standard laboratory protocol includes 500ng of HA-encoding plasmid for H3 PV production. In order to ensure that the quantity of HA was not a limiting factor in the production of Guang11, 1µg of pI.18-Guang11 H3 plasmid was added to the transfection, alongside 250ng of TMPRSS2 or HAT protease plasmid. The increased quantity of HA (with TMPRSS2) did improve the PV titre compared to delta env control (p = 0.037) (Figure 45). However, the titre did not reach the target of $2x10^7$ RLU/ml for use in PVNAs.

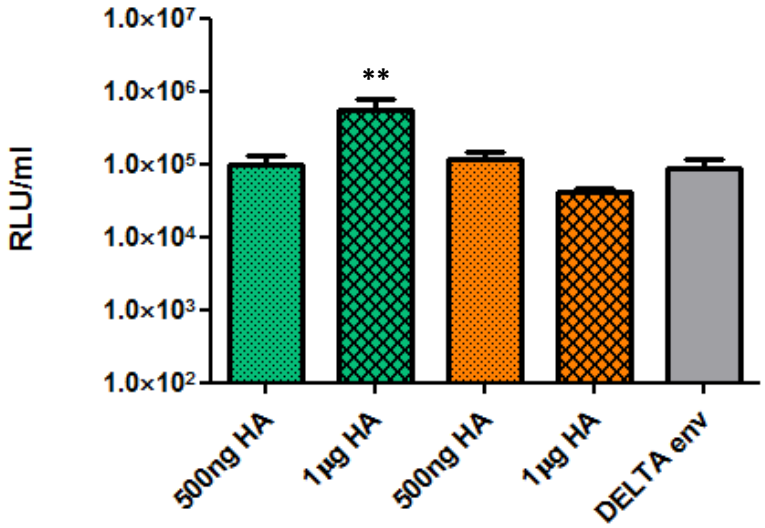


Figure 45. Testing A/canine/Guangdong/3/2011 H3-only PV production using different quantities of pI.18-HA encoding plasmid, in the presence of 250ng TMPRSS2 (green) or 250ng HAT (orange) protease plasmids. Mean PV titres are reported in relative luminescent units (RLU/ml) based on eight replicates with error bars indicating +SEM. ** Indicates a significant difference (p < 0.05) determined by an unpaired T-test.

HEK293T/17 cells are readily amenable to transfection and are therefore a common choice for pseudotype virus production and titration. PV entry into a target cell depends on the surface glycoprotein present on the PV and the receptors expressed on the target cell membrane. Therefore, the titre of a PV is dependent on the target cell line used in the titration assay. Here, a canine cell line (A72 canine tumour of the thigh – Chapter 2 Methods section 2.2.1) was trialled alongside the standard HEK293T/17 as a target cell line for both Colo06 and Guang11 canine influenza PVs. Colo06 was successfully titrated using both A72 and HEK293T/17 cell lines and there was no significant difference between the titres (p = 0.187). Whereas, Guang11 H3-only produced a lower titre when titrated on A72 cells compared with the standard HEK293T/17 cells (Figure 46). Therefore, in the case of canine PVs, infectivity was not increased by changing to a target cell line derived from the host species of the influenza virus strain under study.

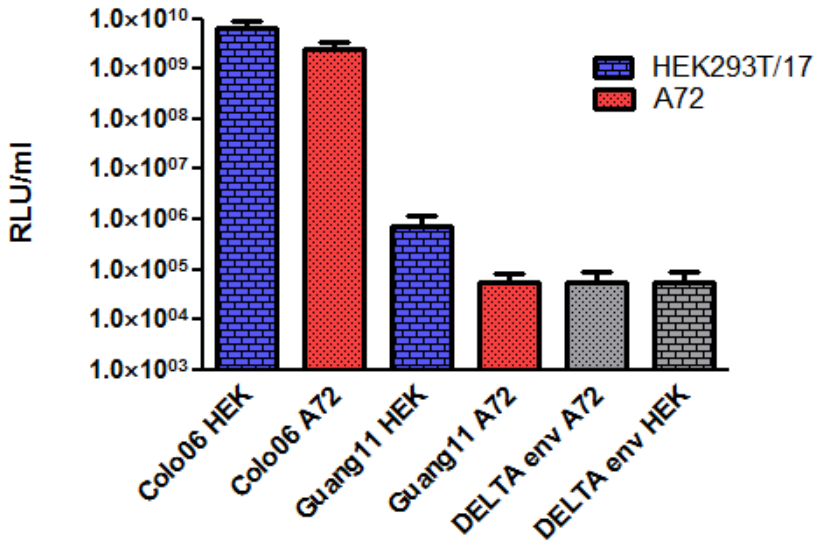


Figure 46. Titration of A/canine/Colorado/30604/2006 H3 PV (Colo06) and A/canine/Guangdong/3/2011 H3 PV (Guang11) using two different target cell lines; HEK293T/17 (HEK in blue) and A72 (red). Delta env negative control was also titrated on both cell lines. Mean PV titres are reported in relative luminescent units (RLU/ml) based on eight replicates with error bars indicating +SEM.

6.3.2 Neutralisation of A/canine/Colorado/30604/2006 H3 PV

Due to a lack of being able to source canine serum for testing against the A/canine/Colorado/30604/2006 H3 PV, a PVNA was set up using a known positive equine H3 polyclonal serum (Methods section 6.2.3): Figure 47 shows the neutralisation curve of the PVNA. There is strong evidence of cross-reactivity between the equine serum and the canine PV with a mean IC_{50} of 57,047.

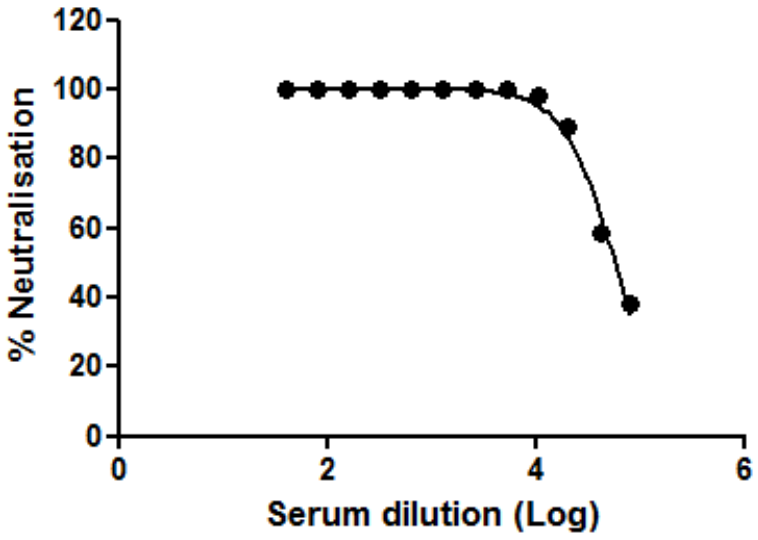


Figure 47. Percentage neutralisation from PVNA of A/canine/Colorado/30604/2006 H3 PV against a known positive equine H3 polyclonal serum. Each data point in the two-fold serial dilution, is a mean of triplicate repeats.

6.3.3 HA-mutant canine influenza PVs for use in HA binding assay

Canine influenza PVs bearing HA proteins containing specific mutations were produced and used to investigate the impact on virus-host cell interactions. An equine Nm/5/03 wild type (WT) HA gene was mutated to incorporate two conserved mutations, independently, that are thought to have contributed to the transmission of the H3N8 virus to dogs (W222L and I328T) (Collins *et al.*, 2014). The mutations were confirmed by sequence analysis. Following this, the two mutant HA-encoding plasmids were used to produce PVs. Mutant W222L did not form a functional PV, based on a comparison to Delta env control (p = 0.491). On the other hand, mutant I328T was successfully produced (Figure 48).

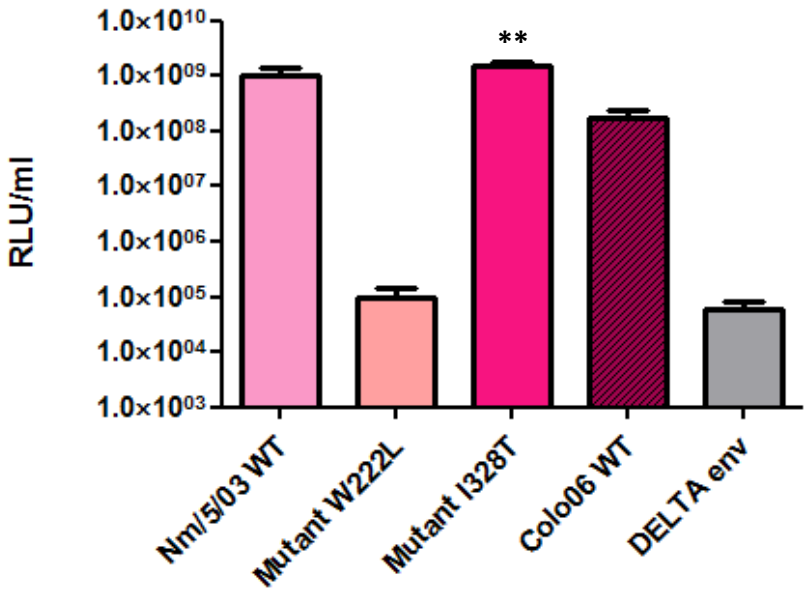


Figure 48. Titration of A/equine/Newmarket/5/2003 (Nm/5/03 WT), mutant W222L, mutant I328T and A/canine/Colorado/30604/2006 H3 (Colo06 WT) PVs. Mean PV titres are reported in relative luminescent units (RLU/ml) based on eight replicates with error bars indicating +SEM. ** Indicates a significant difference ($p < 0.05$) determined by an unpaired T-test.

Although mutant W222L PV was not functional, both mutant PVs were taken forward into a HA-binding assay alongside the Nm/5/03 WT and Colo06 WT PVs previously generated to ascertain any differences in binding of viral particles to sialic acid receptors on target cells. Three different types of red blood cells (RBCs) were used; derived from guinea pig, chicken and sheep species (Figure 49). PVs were serially diluted (two-fold) and the lowest virus titre that caused agglutination of the RBCs was recorded. A Nm/5/03 WT virus was used as a positive control for the assay and it was evident that the whole virus caused agglutination of RBCs at a much lower concentration than the equivalent Nm/5/03 WT PV. The Nm/5/03 WT PV caused agglutination of guinea pig and chicken red blood cells, indicating the presence of HA on the PV surface. The non-functional W222L mutant served as a negative control and did not cause agglutination of RBCs from any species, as expected. The mutant I328T and Colo06 WT, also, did not cause any agglutination of RBCs even at the highest concentration of virus particles (Table 23). Therefore, it was not possible to analyse any difference in receptor binding that could be attributed to the incorporated mutations using this assay.

Table 23. Virus titres (the lowest dilution at which the pseudotyped virus agglutinates RBCs) obtained in HA binding assays using three sources of red blood cell; guinea pig, chicken and sheep. A two-fold serial dilution of virus was carried-out. Nm/5/03 wild type (WT) virus was used as a positive control alongside H3 PVs; Nm/5/03 WT, mutant W222L, mutant I328T and Colo06 WT.

Red Blood Cell Type	Virus				
	Nm/5/03 WT	**Nm/5/03 WT PV**	**Mutant W222L PV**	**Mutant I328T PV**	**Colo06 WT PV**
A) Guinea pig	128	16	< 2	< 2	< 2
B) Chicken	128	16	< 2	< 2	< 2
C) Sheep	64	< 2	< 2	< 2	< 2

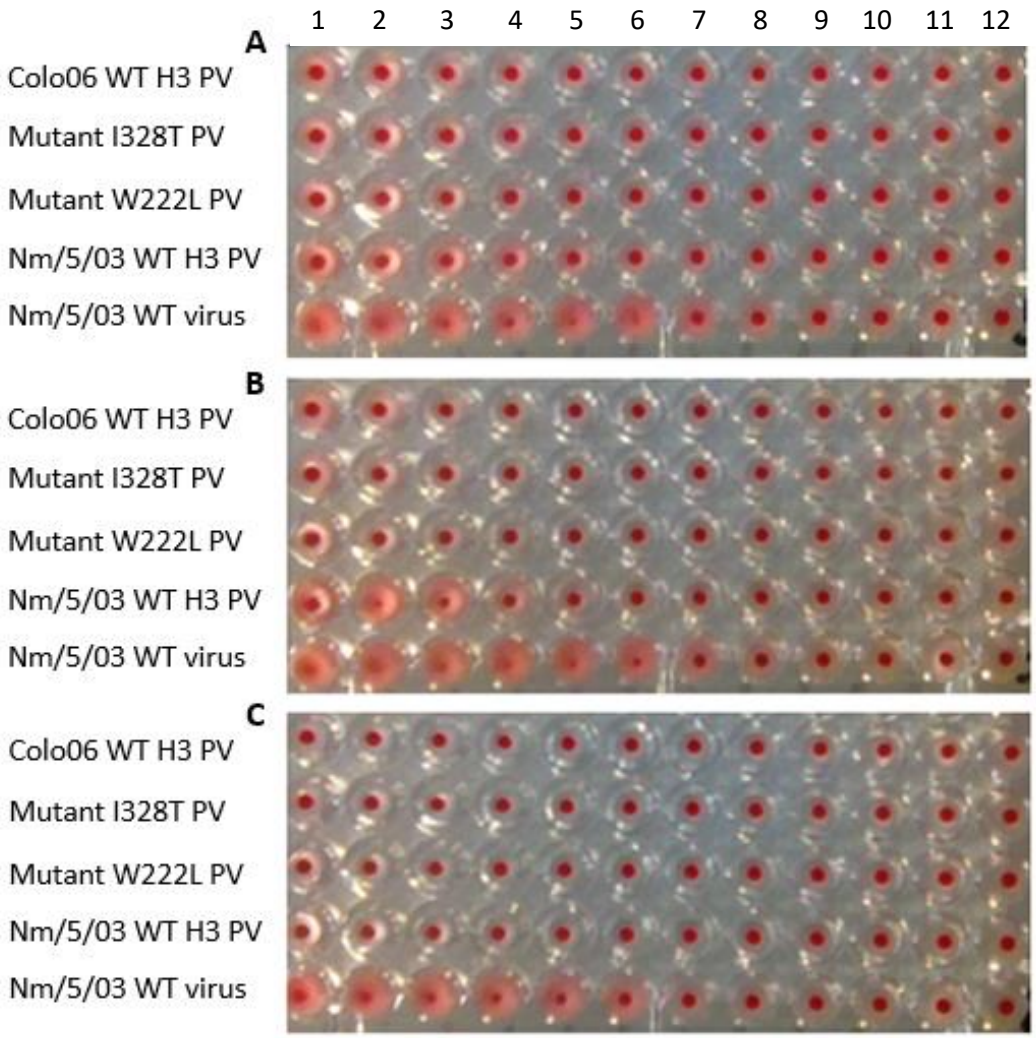


Figure 49. Illustration of haemagglutination (HA) binding assay results using three different sources of red blood cell; A) guinea pig, B) chicken and C) sheep. A two-fold serial dilution of each virus was carried-out beginning in well 1 (1/2 dilution).

6.3.4 Production of a seal influenza PV

Firstly, the A/harbor seal/Massachusetts/1/2011 H3 gene was successfully subcloned into the pI.18 expression plasmid (Figure 50) and used to produce the seal H3 influenza PV. TMPRSS2 and HAT proteases were used in the standard transfection protocol (Chapter 2 Methods section 2.3.1.1) and both were thus clearly successful in cleaving the HA, producing high titre PVs (Figure 51).

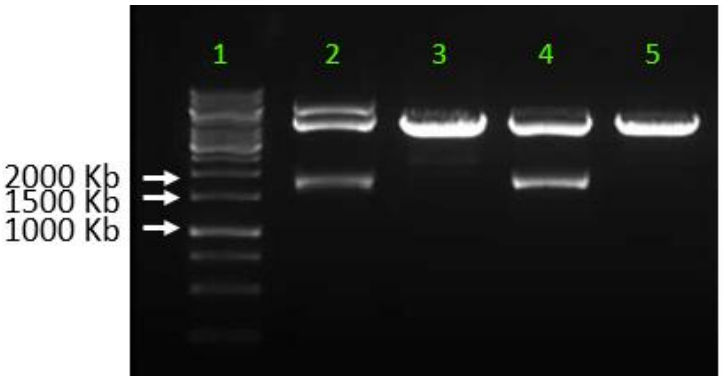


Figure 50. Plasmid digest of pI.18-HA A/harbor seal/Massachusetts/1/2011 H3 using restriction enzymes *KpnI* and *SalI*. Lanes 2 and 4 indicate positive clones. Negative control (lane 5) is pI.18 digested with *KpnI* and *SalI*.

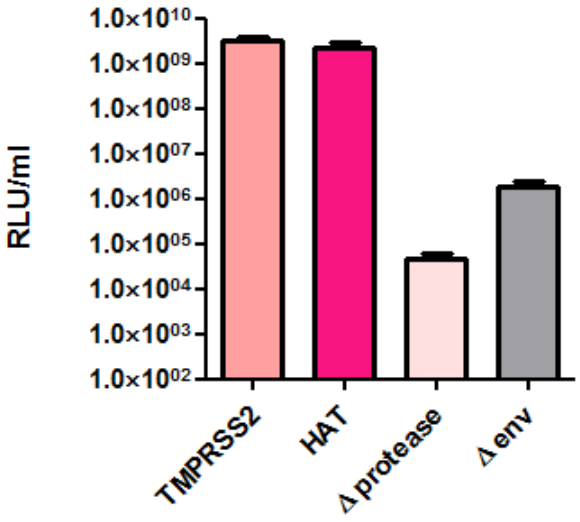


Figure 51. Titration of A/harbor seal/Massachusetts/1/2011 PVs produced with TMPRSS2 and HAT proteases. Δ protease and Δ env negative controls are included. Mean PV titres are reported in relative luminescent units (RLU/ml) based on eight replicates with error bars indicating +SEM.

PVs were then used to assay serum samples taken from wild seals caught in the Baltic Sea (BS) and Caspian Sea (CS) for neutralising antibodies targeting H3 and H10 subtype influenza HAs (Table 24). None of the BS samples were positive for neutralising antibodies against either H3 or H10 PVs. Nine CS samples were positive by H3 PVNA and three via H10 PVNA. All H10 positives were also H3 positive, but three some samples only showed positivity with H3. Furthermore, known positive H3 (equine) and H10 (chicken) sera were assayed against H10 and H3 PVs, respectively, to confirm that the results were subtype specific, not due to cross reactivity (Table 24). The two PVNA experiments showed consistency, with all positives seen in the first assay also positive in the second. ELISA results showed a similar pattern to the positive and negative PVNA results, with the exception of two H3 PVNA positives (CS16 & 22) that were negative by ELISA (Table 24).

Table 24. PVNA results of seal serum samples assayed against A/harbor seal/Massachusetts/1/2011 H3 and A/chicken/Germany/N49 H10 PVs (blue and purple respectively). ELISA results reported as percentage inhibition and highlighted in green. Colour gradient indicates strength of response, whereby the darkest shade represents the strongest positive response. '<' indicates IC_{50} <400 (IC_{50} reported as the reciprocal serum dilution range that leads to 50% neutralisation.) BS – Baltic Sea, CS – Caspian Sea.

		IC_{50} against H3 and H10 PVs			
SERUM SAMPLE	H3 ELISA (% inhibition)	H3 (expt 1)	H3 (expt 2)	H10 (expt 1)	H10 (expt 2)
BS1	0	<	<	<	<
BS2	0	<	<	<	<
BS3	0	<	<	<	<
BS4	0	<	<	<	<
BS5	0	<	<	<	<
CS2	19.9	<	<	<	<
CS9	9.0	<	<	<	<
CS14	33.9	800 - 1600	400 - 800	<	<
CS15	89.4	<	<	<	<
CS16	11.5	~ 800	400 - 800	<	400 - 800
CS17	36.8	<	<	<	<
CS20	75.9	1600 - 3200	1600 - 3200	800 - 1600	800 - 1600
CS21	18.8	<	<	<	<
CS22	17.5	400 - 800	<	<	<
CS23	75.5	400 - 800	<	<	<
CS25	4.9	<	<	<	<
H3 Positive	N/A	3200 - 6400		<	
H10 Positive	N/A	<		>6400	
FBS Negative	N/A	<		<	

Using the A/harbor seal/Massachusetts/1/2011 H3 PV, further PVNAs were set up to analyse the neutralising antibody response in serum samples collected in the Pacific Ocean. Only one sample, number eight, out of the 30 samples was negative in both PVNAs against the seal H3 PV (Table 25). Samples 21, 25, 27, 28 and 30 had low IC_{50} titres in the first PVNA but tested negative in the second. Overall, there was a range of IC_{50} values across the set of serum samples but majority gave strong positive results against the H3. Surprisingly, all samples tested negative (triplicate repeats) in the same ELISA as used above.

Table 25. IC_{50} results obtained from a PVNA using Pacific Ocean seal serum samples against A/harbor seal/Massachusetts/1/2011 H3 PV. H3 (equine) positive and fetal bovine negative control sera were included. ELISA results illustrated in green (all samples negative). Colour gradient indicates strength of response, whereby the darkest shade represents the strongest positive response. * Indicates an IC_{50} value of < 100.

SERUM SAMPLE	IC_{50}	
	Assay 1	Assay 2
1	1600 - 3200	1600 - 3200
2	100 – 200	100 – 200
3	400 – 800	800 – 1600
4	200 – 400	100 – 200
5	200 – 400	400 – 800
6	400 – 800	800 – 1600
7	800 - 1600	1600 - 3200
8	*	*
9	800 – 1600	800 – 1600
10	400 – 800	800 – 1600
H3 Positive	3200 - 6400	>6400
FBS Negative	*	*

SERUM SAMPLE	IC_{50}	
	Assay 1	Assay 2
11	800 - 1600	400 – 800
12	800 – 1600	800 – 1600
13	800 – 1600	200 - 400
14	1600 – 3200	200 - 400
15	800 – 1600	800 – 1600
16	400 – 800	200 - 400
17	400 – 800	200 - 400
18	200 - 400	200 - 400
19	400 – 800	800 – 1600
20	400 – 800	800 – 1600
H3 Positive	3200 - 6400	>6400
FBS Negative	*	*

SERUM SAMPLE	IC_{50}	
	Assay 1	Assay 2
21	400 - 800	*
22	1600 – 3200	400 – 800
23	400 – 800	400 – 800
24	400 – 800	800 - 1600
25	400 - 800	*
26	200 – 400	100 – 200
27	200 – 400	*
28	100 - 200	*
29	800 – 1600	3200 – 6400
30	100 – 200	*
H3 Positive	>6400	>6400
FBS Negative	*	*

6.3.5 Production of different HA/NA combination PVs

Attempts were made to produce PVs bearing different combinations of HA and NA to investigate the effect of multiple surface glycoproteins on PV titre and the potential to use the PVs as reassortment prediction tools. All combinations of HA and NA trialled currently circulate in nature, predominantly in avian hosts. However, in this study the compatibility of HA and NA from different species was investigated. Initially, all available NA-encoding plasmids were co-transfected with A/equine/Richmond/1/2007 H3 or A/Viet Nam/1194/2004 H5 (Table 26). All NA plasmids facilitated the production of high titre H5NA PVs, which confirmed that all NA plasmids were functional, expressing NA. Furthermore, with the exception of N2, all NA plasmids produced higher titres than the standard addition of exogenous NA (ExNA) for H5 PVs (Figure 52). It is noteworthy that the N2 of human origin produced viable PVs that were significantly higher in titre than the H5 delta NA control (p = <0.001). Whereas this was not the case for H3; the human N2 plasmid did not facilitate the production of an H3N2 PV above the level of H3 delta NA (p = 0.462 and p = 0.798 respectively). Only the H3N1 combination produced a titre that was statistically comparable to ExNA (p = 0.505). The N3, N4 and N9 plasmids produced PVs with significantly increased titres, compared to H3 delta NA, but none were comparable to the highest titre using ExNA. Interestingly, the matching strain HA and NA (H3N8) was functional (p = <0.01) but did not produce the highest titre PV.

Table 26. Combinations of HA and NA (4:1) PVs generated. The matched equine subtype that is currently circulating is highlighted in purple. Functional PVs were defined by a significantly higher titre than a delta NA control (Mann-Whitney test, $p \leq 0.01$) are highlighted green. Non-functional PVs are highlighted red.

HA Origin	NA Origin	Combination
A/equine/Richmond/1/2007 (H3N8)	A/chicken/Italy/1067/1999 (H7N1)	**H3 N1**
A/equine/Richmond/1/2007 (H3N8)	A/Udorn/307/1972 (H3N2)	**H3 N2**
A/equine/Richmond/1/2007 (H3N8)	A/chicken/Pakistan/34669/1995 (H7N3)	**H3 N3**
A/equine/Richmond/1/2007 (H3N8)	A/turkey/Ontario/6118/1968 (H8N4)	**H3 N4**
A/equine/Richmond/1/2007 (H3N8)	A/equine/Richmond/1/2007 (H3N8)	**H3 N8**
A/equine/Richmond/1/2007 (H3N8)	A/avian/Shanghai/2/2013 (H7N9)	**H3 N9**
A/Viet Nam/1194/2004 (H5N1)	A/chicken/Italy/1067/1999 (H7N1)	**H5 N1**
A/Viet Nam/1194/2004 (H5N1)	A/Udorn/307/1972 (H3N2)	**H5 N2**
A/Viet Nam/1194/2004 (H5N1)	A/chicken/Pakistan/34669/1995 (H7N3)	**H5 N3**
A/Viet Nam/1194/2004 (H5N1)	A/turkey/Ontario/6118/1968 (H8N4)	**H5 N4**
A/Viet Nam/1194/2004 (H5N1)	A/equine/Richmond/1/2007 (H3N8)	**H5 N8**
A/Viet Nam/1194/2004 (H5N1)	A/avian/Shanghai/2/2013 (H7N9)	**H5 N9**

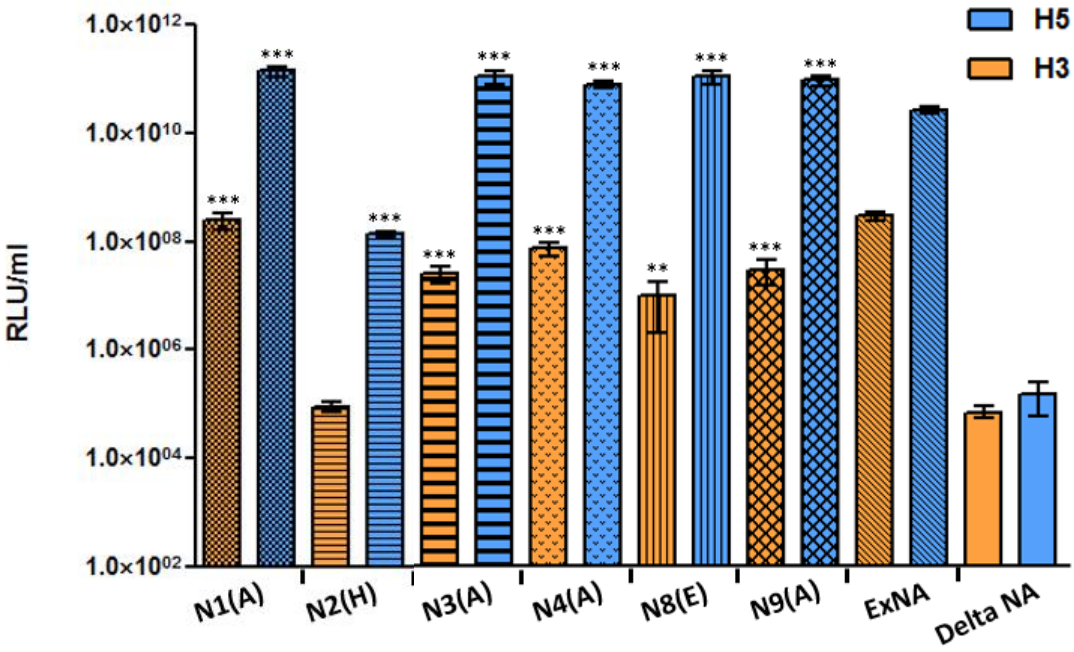


Figure 52. A/equine/Richmond/1/2007 H3 (orange) and A/Vietnam/1194/2004 H5 (blue) mean PV titres generated by co-transfection with different NA plasmids (Table 22 contains NA strains). Species origin of NA denoted by; A – avian, C – canine, E – equine, and H – human. An exogenous source of NA (ExNA) was added 24hours post transfection as a positive control and Delta (without) NA was generated as a negative control. PV titres reported in relative luminescent units (RLU/ml), +/- SEM of eight replicates. Statistically significant differences (Mann-Whitney test) between mean RLU/ml of each HA/NA combination PV and the Delta NA control are indicated by ** = p<0.01; *** = p<0.001.

Further combinations of equine, canine, phocine and human HA and NAs were trialled with a view for reassortment potential (Table 27), based on the concept of HA/NA evolution whereby not all combinations are compatible to generate infectious virus particles. Surprisingly, the matching human H3N2 strain did not generate a functional PV (p = 0.07). Novel combinations of seal H3 with human N2 and human H3 with equine N8 also did not generate functional PVs. Interestingly however, one out of the two H1 human and N8 equine PV combinations was successfully generated (South Carolina; p = 0.005). The H3 canine and N8 equine combination was also successful (Figure 53).

Table 27. Combinations of HA and NA (4:1) PVs generated. The matched human subtypes that is currently circulating is highlighted in purple. Functional PVs were defined by a significantly higher titre than the delta env control (Mann-Whitney test, $p \leq 0.01$) are highlighted green. Non-functional PVs are highlighted red.

PV	HA Origin	NA Origin	Combination
1	A/South Carolina/1/1918 (H1N1)	A/equine/Richmond/1/07 (H3N8)	**H1 N8**
2	A/Texas/05/2009 (H1N1)	A/equine/Richmond/1/07 (H3N8)	**H1 N8**
3	A/Udorn/307/1972 (H3N2)	A/Udorn/307/1972 (H3N2)	**H3 N2**
4	A/harbor seal/Massachusetts/1/2011 (H3N8)	A/Udorn/307/1972 (H3N2)	**H3 N2**
5	A/Udorn/307/1972 (H3N2)	A/equine/Richmond/1/07 (H3N8)	**H3 N8**
6	A/canine/Colorado/30604/2006 (H3N8)	A/equine/Richmond/1/07 (H3N8)	**H3 N8**

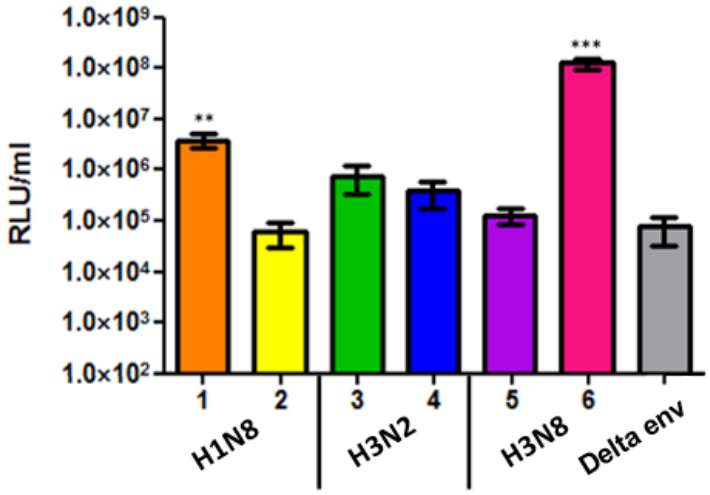


Figure 53. H1N8, H3N2 and H3N8 combination PVs. Mean PV titres generated by co-transfection with different HA and NA plasmids (Table 27 contains HA and NA strains for PVs 1-6). Delta envelope (env) was generated as a negative control. PV titres reported in relative luminescent units (RLU/ml), +/- SEM of eight replicates. Statistically significant differences (Mann-Whitney test) between mean RLU/ml of each HA/NA combination PV and the Delta env control are indicated by ** = p<0.01; *** = p<0.001.

The three highest titre H3NA PVs were taken forward into a preliminary PVNA to establish whether PVs incorporating both HA and NA proteins on their surface can be neutralised. An H3 positive serum from a horse that had been serially vaccinated with several H3 equine influenza strains was used (Scott *et al.*, 2012). All PVs were neutralised and those with both HA and NA resulted in higher IC_{50} values compared with the H3-only PV produced with exogenous NA; H3N1 showed a 2-fold increase, H3N3 was the greatest increase by 11-fold and H3N4 increased by 3-fold. Hence the presence of NA on the surface of the pseudotyped virus increased neutralisation compared to using an exogenous source of NA.

Table 28. PVNA IC_{50} values obtained from an H3 positive equine polyclonal serum against HA/NA combination PVs. Fold-difference to the equine H3 only PV (produced with exogenous neuraminidase) are stated for all HA/NA combinations. PV titres greater than 2 x 10^7 RLU/ml were used in the assay.

PV	IC_{50}	Fold-difference
Equine H3 + Avian N1	92,463	1.93
Equine H3 + Avian N3	541,169	11.32
Equine H3 + Avian N4	156,340	3.27
Equine H3 only	47,792	1.00

6.3.5.1 Neuraminidase activity assays

In order to provide a potential explanation for differences in PV titre with different combinations of HA and NA, NA activity assays were carried-out. Initially, PVs were diluted to an equal titre (based on the lowest titre PV) and NA activity was measured in relative fluorescent units (RFUs) (Table 29). Using diluted PVs, only supernatant samples of the H3 and H5 PVs produced with exogenous NA gave a high level of activity and the H3N9 PV showed a low level of activity. All other PVs showed minimal or no NA activity, at the same level as the negative control. The NA activities using neat PVs were higher for all combinations apart from the N2 PV. However, the titres differed when using neat PVs, and therefore the activity of the NA can only be compared in cases where PV titre could be normalised i.e. the same titre: this is true for H3N3 & H3N9 and H5N3 & H5N8. The levels of NA activity are similar in these cases, indicating that the titre of a PV could be affected by the level of NA activity. This is also supported by the low titre H3N2 PVs (Table 29) exhibiting low NA activity.

Table 29. Neuraminidase activity levels of H3NA and H5NA combination PVs produced with plasmid-encoding NAs or an exogenous NA (ExNA) control. PV titres are reported in relative luminescent units/ml (RLU/ml). Activity was measured in relative fluorescent units (RFUs).

	H3 PVs			H5 PVs		
NA	**Titre (RLU/ml)**	**NA Activity (RFU)**		**Titre (RLU/ml)**	**NA Activity (RFU)**	
		Neat	**Diluted**		**Neat**	**Diluted**
N1	2.5×10^8	216	9	8.4×10^{10}	393	5
N2	$< 10^5$	7	-	1.4×10^8	6	5
N3	2.5×10^7	10	8	1.0×10^{11}	149	5
N4	7.1×10^7	11	6	7.6×10^{10}	24	-
N8	9.8×10^6	9	8	1.1×10^{11}	598	6
N9	2.9×10^7	28	17	9.0×10^{10}	110	-
ExNA	2.8×10^8	871	560	2.6×10^{10}	1073	15
Negative	N/A	5	5	N/A	5	6

6.4 Discussion

Canine influenza H3 PVs

Novel expression vectors for use in mammalian cells are continuously being developed and can be purchased from numerous companies. Genscript® provided an opportunity to test an alternative expression vector, pDREAM2.1, to our standard pI.18 plasmid in our lentiviral-based pseudotype virus system. pDREAM2.1 was engineered with several promoters in order to function within bacteria, insect cells and mammalian cells. Our standard protocol plasmid, pI.18, contains a human cytomegalovirus promoter (CMV) that drives expression of the HA gene in HEK293T/17 cells. The CMV promoter is also present in pDREAM2.1, however, the plasmid did not facilitate expression of either canine HA genes (Colo06 or Guang11) to a level that generated functional pseudotyped viruses and the plasmid was therefore disregarded from further use.

The Colo06 pI.18-H3 encoding plasmid, alongside the core gag-pol plasmid (p8.91) and luciferase reporter (pCSFLW), facilitated the generation of Colo06 H3-only PV but Guang11 pI.18-H3 did not. Cleavage of HA is essential for infection by an influenza virion and therefore optimising the protease is a strategy employed to rescue pseudotype virus production. TMPRSS2 and HAT are commonly used to cleave H3 subtypes but did not facilitate the production of functional Guang11 PV. Furthermore, the cleavage site in Guang11 is identical to the seal H3 (A/harbor seal/Massachusetts/1/2011) cleavage site, which was successfully cleaved by TMPRSS2 and HAT. Alternative proteases were therefore trialled in an attempt to produce functional Guang11 H3-only PV. TMPRSS4 has been shown to successfully cleave H3 subtypes (Bertram *et al.*, 2010a) however, for Guang11 H3, cleavage was not successful. Data published on TMPRSS3 and TMPRSS6 proteases reported no HA cleavage (Kühn *et al.*, 2016) and our results were in agreement; neither TMPRSS3 or 6 increased PV titres above that of the delta envelope control. KLK5, a protease found in the blood, has been shown to cleave HA (Hamilton & Whittaker, 2013) but was also not successful for cleaving Guang11 H3. It is possible that amino acid 321I, which is different to 321V contained within other H3s that have been successfully pseudotyped, impacts the HA protein folding and furthermore affects cleavage. Alternatively, cleavage of few PVs could be occurring, yet a combination of low-expression of the H3 gene and lack of binding to target cells could be causing a low titre PV to be

generated. Functional H3-only equine, canine and phocine PVs were produced with a standard 500ng of HA-encoding plasmid DNA, however this was not successful with Guang11 H3. Increasing the quantity of HA could increase the abundance of HA protein on the surface of the PV, which would increase the likelihood of PV virus binding to target cells, assuming that cleavage of the HA has been successful. Doubling the quantity of the HA-encoding plasmid did increase the titre of the Guang11 PV, however, the titre did not reach $2x10^7$ RLU/ml, which is the desired input for a PVNA. Future work should include increasing the quantity of HA-encoding plasmid and carrying-out western blot analysis to confirm a) expression of the HA protein b) presence of cleaved HA and c) optimised vs non-codon optimised genes.

A study based on VSV-pseudotyped influenza viruses illustrated that matching HA and NA proteins enhance HA-fusion and furthermore entry of PVs compared to HA-only pseudotypes (Hsu *et al.*, 2015). Independently, we also demonstrated that it is possible to generate HA and NA lentiviral pseudotypes (Scott *et al.*, 2016) and decided to investigate if the presence of the native A/canine/Guangdong/3/2011 N2 protein would increase PV titre. Sequence analysis of the subcloned N2 gene in pI.18 uncovered two issues; firstly, the orientation of the gene was reversed as a result of incorrect cloning and secondly, a stop codon was present mid-way through the gene. These issues have since been rectified but the resulting Guang11 H3 and NA 'matching' PV was still non-functional (Martin Mayora-Neto pers. comm.).

Another approach to trouble-shooting the non-functional canine Guang11 PV was to change the target cell line from human to canine origin. HEK293T/17 cells are permissive to influenza pseudotyped viruses, although not thus far for Guang11. Therefore, a change in receptor composition on the target cell surface may be required for GuangH3-mediated entry. A canine cell line A72 was trialled without success. MDCK (Madin-Darby Canine Kidney) cells are commonly used in influenza virus studies and would be a good candidate cell line for future studies (Hsu *et al.*, 2015; Lin *et al.*, 2016).

Agglutination of RBCs in binding assays has been demonstrated with highly pathogenic H5N1 lentiviral pseudotypes and furthermore, single amino acid changes have been shown to alter the binding specificity of PVs (Tang *et al.*, 2016). Here, we adopted the same approach, with A/equine/Newmarket/5/03 wild-type H3 PV and introduced two separate canine influenza-associated mutations. All three PVs were added to RBCs to

ascertain any differences in binding. However, it was not possible to analyse differences in receptor binding because the mutants did not cause the RBCs to agglutinate. The W222L mutant did not form a functional PV and therefore a lack of HA on the PV surface would explain a lack of binding. On the other hand, the I328T mutant generated a higher titre PV than the A/equine/Newmarket/5/03 and A/canine/Colorado/30604/2006 H3 wild-type PVs, yet did not cause agglutination of RBCs. It is possible that the canine influenza does not cause agglutination of guinea pig, chicken or horse RBCs. Haemagglutination inhibition assays have successfully been conducted using turkey RBCs, therefore this assay should be repeated with turkey RBCs (Anderson *et al.*, 2012b; Crawford, 2005).

Seal influenza H3 PV

Following successful generation of the A/harbor seal/Massachusetts/1/2011 H3 PV, serosurveillance of wild seal sera was conducted using the PVNA. Sera were collected from seals in the Baltic and Caspian Seas and were originally tested in a competitive ELISA (Sasan Fereidouni and Elke Starick unpublished data), which detects antibodies against the nucleocapsid protein of the influenza virus. The ELISA results were overall in agreement with the PVNA results, whereby positive ELISA results were also positive in the PVNA. However, there were two exceptions with samples CS15 and CS23, which tested as strong positives in the ELISA but were considered as negative and 'weakly positive' in the PVNA, respectively. This was the first serosurveillance study of seal sera conducted with the PVNA and therefore the negative threshold of samples needs to be considered. A threshold for equine serum negative for specific influenza antibodies was originally established on a limited set of samples (Scott *et al.*, 2012) but whether this threshold is applicable for seal sera needs to be addressed. Negative ELISA results equated to an IC_{50} between 400 and 800, which is higher than the threshold of 80 for equine samples. It is possible that non-specific binding of proteins in the seal sera elevated the IC_{50} result.

Another serosurveillance study was conducted on a set of wild seal sera, collected from pups in the Pacific Ocean. Interestingly, ELISA results were negative but IC_{50} results from the PVNA were considered to be positive, based on the original equine threshold of IC_{50} below 80. It would be interesting to PVNA test this set of sera from the Pacific Ocean against another influenza subtype to establish if the higher, than expected, level of neutralisation is seen against other subtypes. The PVNA has been reported with higher

levels of sensitivity compared to other serological assays, such as single radial haemolysis (Scott *et al.*, 2012). One of the advantages of the PVNA is that small quantities of sera are required, however, this means that sera tested in PVNAs are typically at lower concentrations than in other assays such as the ELISA. Therefore the 'positive' results cannot be attributed to a higher concentration of serum. It would be beneficial to carryout ELISA and PVNAs with the same dilution of sera in order to understand equivalent read-outs for the different assays. Unfortunately, with the limited serum sample volumes in this study, it was not possible to carry-out the PVNA at the same concentration as the ELISA. Another point to consider is that the ELISA employed here detects antibodies against the nucleocapsid protein whereas the PVNA detects neutralising antibodies against HA, therefore a direct comparison between the assays would not be possible. An ELISA that detects antibodies against H3 HA would be beneficial to aid correlation enquires.

HA and NA PVs

A proof of principle study in chapter 3 demonstrated that it is possible to generate an equine H3N8 influenza pseudotype, bearing both HA and NA surface glycoproteins. In this chapter, the concept was extended to generate different combinations of HA and NA PVs, how this affects PV titre and if mismatched HA&NA combinations are functional. One PV study has already shown that matching HA and NA proteins enhanced HA-fusion and furthermore entry of PVs compared to HA-only pseudotypes, which is due to the positive effect on HA-trafficking through the producer cell to the cell surface and thus increasing the amount of HA on the PV surface (Hsu *et al.*, 2015). However, there is also evidence to suggest that adding either exogenous or endogenous NA yields similar PV titres (Su *et al.*, 2009). Here, the production of H5 PVs was enhanced by the presence of endogenous NA compared to the bacterial source of exogenous NA, which is in agreement with Tsai and colleagues (2009), whereas for H3 PV production exogenous NA generated the highest PV titre. Interestingly, the matching combination of A/Udorn/307/1972 H3 and N2 was not functional and neither were other combinations that incorporated the N2; A/equine/Richmond/1/2007 H3 and A/harbor seal/Massachusetts/1/2011 H3. The N2-encoding plasmid was transfected alongside a H5-encoding plasmid and formed a functional PV, therefore the plasmid was functional. However, the resulting H5N2 PV was 2 logs lower in titre than other H5 and NA PVs. A neuraminidase activity assay was carried-

out and found that the N2 activity was lower that all other NAs, which provides an explanation for the resulting low titre and non-functional N2 PVs. A reassortment study with the H9 subtype also found that N2 activity was lower than N1 and N3 (Yan *et al.*, 2016).

Although the transfection ratio of HA:NA is representative of the 4:1 ratio on an influenza virion, we have not established the distribution pattern of the proteins on the PV surface. The expression of both proteins may not be equal and therefore different quantities of each embedded in the membrane could be impacting PV titre and furthermore neutralisation assays. Neutralisation assays with H3NA PVs (H3N1, H3N3 and H3N4) were trialled and the neutralising antibody responses against the PVs with NA expressed were greater than against the HA-only PV produced with exogenous NA, which initially suggested the H3 polyclonal serum (serially vaccinated equine) had antibodies against NA. However, NA antibodies are well-known to facilitate the release of progeny virions and therefore this should not impact the neutralisation of PVs because they do not replicate and egress from the target cells. Therefore, it is more likely that the HA & NA PVs have less HA on the surface and thus the anti-HA antibody activity is more efficacious in neutralising the PV. For future work, western blots need to be carried out to determine the level of HA expression in PVs produced with exogenous versus endogenous sources of NA.

7. CONCLUSIONS AND FUTURE WORK

The pseudotype platform has been employed for different purposes in this PhD study (thesis chapters 4–6); for use in a vaccine efficacy trial, dissecting antigenic drift using mutant PVs, serosurveillance studies and to assess its potential use in studying reassortment events, respectively. Much has been written and debated concerning the advantages and disadvantages of different serological assays, but the most important point is to choose the appropriate assay for the application.

Employing the PVNA in an equine influenza vaccine efficacy trial is the first known study of its kind and the results demonstrated potential for application in future commercial studies the field (Romain Paillot pers. comm.). The results of the PVNA correlated well with SRH data, which is used by the OIE as a standard assay for equine influenza vaccine testing (OIE, 2008). There is a strong correlation between post-vaccination protective immunity and antibody detection in the SRH assay, which is why it is a favoured choice for testing vaccine efficacy. The quantification of the two assays differs however; the SRH assay generates a continuous read-out measurement e.g. 123 mm^2, whereas the PVNA is based on a two-fold serial dilution series. GraphPad Prism software enables a neutralisation curve to be plotted on a continuous scale (serum concentration) such that the IC_{50} calculated is not restricted to an exact 'titre', but will fall somewhere in the range of two serial dilution points. The results of a PVNA could therefore be presented in three ways i.e. the IC_{50} value, a titre range (in which the IC_{50} falls) or the titre value at which at least 50% neutralisation is reached. For the purpose of vaccine efficacy testing using SRH, minimal values for protection have been defined (Mumford & Wood, 1991). Further work needs to be conducted to try and determine a protective level of antibody measured by PVNA, as for the SRH, and how these values will be reported. In future studies, data on virus shedding and clinical signs post challenge would be necessary to differentiate between clinical protection and prevention of viral shedding when assessing correlates of protection.

The PVNA was also applied to another investigation. For historical cases where vaccines have failed to protect against emerging equine influenza strains, mutant HA PVs were generated to dissect the changes in immune response and investigate how specific mutations can impact vaccine efficacy. Reactivity of sera (both ferret and equine) against

single mutant PVs were compared with the respective wildtype PV and changes in recognition by antibodies were evident, but how these changes would be reflected in clinical signs is unknown. Future work could investigate the impact of multiple mutations within the HA on the immune response and whether significant changes are observed. A caveat to generating mutant PVs is how the changes can affect production. Due to different PV titres obtained with mutant and wildtype PVs it was essential to have an equivalent RLU input, in order to draw comparisons between mutant and wildtype PVs. In some cases, a mutant PV was not included in an assay due to low titre; future work could implement a lower RLU input threshold. A study has shown that it is possible to reduce the RLU input of PVs to as low as $2.5x10^3$ RLU (Logan *et al.*, 2016), which would allow lower titre mutants to be used in assays. In addition to lowering RLU input, it would be beneficial to implement a $TCID_{50}$ titration assay to ascertain a level of infectious units that are added to an assay (Wright *et al.*, 2009) aiding quantification of PV input. It is also important to consider that, although the HA protein is well documented as the primary target for antibodies, other studies are looking into the effect of other proteins on virus evolution and subsequent immune escape (Murcia *et al.*, 2011). A recent study suggests that NA contributes considerably to protective immunity (Memoli *et al.*, 2016). Future work could involve investigating influenza NA pseudotypes and mutations within this surface glycoprotein.

Novel PVs, such as those derived from canine and phocine (seal) influenza strains have been produced and provide a new platform for serosurveillance and seroepidemiology of these viruses, particularly in the case of wild or feral animals, due to issues with obtaining samples from wild animals during acute infection. It is also possible that canine PVs could be used in future canine vaccine efficacy testing.

In conclusion, pseudotypes have demonstrated to be flexible tools and the PVNA has demonstrated its potential for use in efficacy testing of equine influenza vaccines.

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