

**Investigation of an atypically produced Jeryl Lynn
mumps vaccine**

Sarah Margaret Connaughton
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Imperial College London
Department of Medicine
Section of Virology

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ABSTRACT

Mumps virus is the causative agent of mumps disease, of which humans are the only natural host. Mumps infection is vaccine-preventable. Mumps vaccines are live viruses attenuated by serial passage on cell substrates or in embryonated eggs. They are known to vary in their effectiveness, degree of attenuation and adverse event profile. The Jeryl Lynn mumps virus is the most commonly used vaccine strain. Two related commercial mumps vaccines, which originate from the same attenuated strain (Jeryl Lynn-5) but have different efficacies, were investigated. Jeryl Lynn-Canine Kidney (JL-CK), produced on primary canine kidney cells is less effective than RIT 4385 which is produced on chicken embryo fibroblasts. JL-CK and RIT 4385 could be distinguished by a number of *in vitro* and *in vivo* properties. JL-CK produced heterogeneous, generally smaller plaques than RIT 4385, but gave 100 fold higher viral titres when grown on Vero or MDCK cells and produced a higher degree of hydrocephalus in the neonatal rat brain. Sequencing of the JL-CK virus identified 14 regions of heterogeneity throughout the genome. Plaque isolation demonstrated the presence of five mumps virus variants encompassing these mutations in the JL-CK vaccine. One mutation was associated with a small plaque phenotype, the effects of the others either *in vitro* or *in vivo* were less clear. Only 4 % of the JL-CK vaccine population corresponded with the parent Jeryl Lynn-5 strain. Deep sequencing of JL-CK and virus replicating in cell lines and neonatal rat brains showed that propagation *in vitro* or *in vivo* altered the population dramatically. The data presented in this study suggest that growth of JL-CK in primary canine kidney cells resulted in the selection of a mixture of mumps virus variants that have different biological properties compared to the parent Jeryl Lynn-5 virus.

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ABBREVIATIONS

a	adenine
aa	amino acid
AEFI	adverse events following immunisation
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
CEF	chicken embryo fibroblasts
CMC	carboxymethylcellulose
CPE	cytopathic effect
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotides
d.p.i	days post-infection
EDTA	ethylenediamine tetra acetic acid
ELISA	enzyme linked immunosorbant assay
EMA	European Medicines Agency
F	fusion protein or gene
F ₀	fusion protein inactive precursor
F ₁	fusion protein carboxy-terminal subunit
F ₂	fusion protein amino-terminal subunit
FCS	foetal calf serum
FDA	Food and Drug Administration
g	guanine
GSK	GlaxoSmithKline
HN	haemagglutinin-neuraminidase protein or gene
hr	hour(s)
HRP	horseradish peroxidase
IMS	industrial methylated spirit
JL-2	Jeryl Lynn-2
JL-5	Jeryl Lynn-5

JL-CK	Jeryl Lynn canine kidney
kb	kilo base
kDa	kilo Dalton
l	litre(s)
L	large (polymerase) protein or gene
L-3	Leningrad-3
L-3 SSW	Leningrad-3 Sächsisches Serumwerk
LC-MS/MS	liquid chromatography tandem mass spectrometry
M	matrix protein or gene
mA	milliamps
MAPREC	mutant analysis by PCR and restriction enzyme cleavage
mda-5	melanoma differentiation-associated gene-5
MDCK	Madin Darby canine kidney
MEM	Minimal Essential Medium
MHRA	Medicines and Healthcare products Regulatory Agency
min	minute(s)
ml	millilitre
mM	millimolar
MMR	measles, mumps, rubella
MMRV	measles, mumps, rubella, varicella
MOI	multiplicity of infection
MPC	magnetic particle concentrator
mRNA	messenger RNA
MTS	3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt
MuV	mumps virus
N	nucleoprotein or gene
NIBSC	National Institute for Biological Standards and Control
nm	nanometre(s)
nt	nucleotide
P	phosphoprotein or gene

PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming unit
p.i.	post-infection
PRN	plaque reduction neutralisation
PVDF	polyvinylidene fluoride
RNA	ribonucleic acid
RNP	ribonucleoprotein
RT	reverse transcriptase
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulphate
sec	second(s)
SH	small hydrophobic protein or gene
SII	Serum Institute of India
STAT	signal transducers and activators of transcription
TBE	tris borate ethylenediamine tetra acetic acid
TBS	tris buffered saline
TE	tris ethylenediamine tetra acetic acid
Ter	terminator/stop codon
UK	United Kingdom
μl	microlitre
μM	micromolar
US	United States
UV	ultra violet
VLP	virus-like particle
WHO	World Health Organisation

AMINO ACID ABBREVIATIONS

Alanine	A
Arginine	R
Asparagine	N
Aspartic acid	D
Cysteine	C
Glutamic acid	E
Glutamine	Q
Glycine	G
Histidine	H
Isoleucine	I
Leucine	L
Lysine	K
Methionine	M
Phenylalanine	F
Proline	P
Serine	S
Threonine	T
Tryptophan	W
Tyrosine	Y
Valine	V

CHAPTER 1 – INTRODUCTION

1.1 A brief history of mumps virus

Mumps is one of the earliest human diseases recorded. In his Book of Epidemics, in the 5th century B.C., Hippocrates described an outbreak of mumps on the island of Thasos in the Aegean Sea during the autumn equinox. At the time he described the characteristic swelling of the parotid glands either unilaterally or bilaterally accompanied with a slight fever. He noted that males who presented with parotid swelling would commonly develop swelling of the testes and that mumps disease in females was less common. The cause of mumps disease remained unknown until the 19th century, when in 1908 Granata suggested a filterable pathogen was the etiological agent of mumps, referenced in (Wollstein, 1916). In 1934, Johnson and Goodpasture demonstrated by inoculating filtered saliva from infected patients into the Stensen's duct of rhesus macaques, that the disease was caused by a virus (Johnson and Goodpasture, 1934). The virus could also be transferred between macaques by inoculation with homogenised parotid glands excised from infected animals. Further studies revealed that inoculation with filter-sterilised, bacteria-free homogenised parotid glands from infected macaques caused disease in volunteer children. The saliva from the volunteers who displayed clinical symptoms of mumps was used to inoculate healthy macaques. The animals developed symptoms of mumps. This fulfilled Koch's postulates and led to the conclusion that the virus was the causative agent of mumps disease in humans (Johnson and Goodpasture, 1935).

1.2 Mumps virus taxonomy

Mumps virus (MuV) is an enveloped, non-segmented, single-stranded, negative-sense RNA virus belonging to the genus *Rubulavirus* in the family *Paramyxoviridae* of the order *Mononegavirales*. The *Paramyxoviridae* are further classified into the sub-families *Paramyxovirinae* and *Pneumovirinae*. The *Paramyxovirinae* sub-family are grouped based on the size and shape of their nucleocapsid. They include mumps virus (*Rubulavirus*) measles virus (genus *Morbillivirus*), Sendai virus (genus *Respirovirus*), Newcastle disease virus (genus *Avulavirus*) and Hendra virus (genus *Henipavirus*). The *Pneumovirinae* sub-family contains human respiratory syncytial virus (genus *Pneumovirus*) and human metapneumovirus (genus *Metapneumovirus*).

Members of the *Rubulavirus* genus all display antigenic cross-reactivity and the presence of neuraminidase activity (Lamb and Parks, 2007). Other members of the *Rubulavirus* genus include; parainfluenza virus 5 which was originally called simian virus 5 after its initial isolation from monkey kidney cultures (Chatziandreou et al., 2004), human parainfluenza virus type 2, type 4a and type 4b. Porcine rubulavirus (previously called La Piedad Michoacan Mexico virus), (Lamb and Parks, 2007; Wang et al., 2007) and Menangle virus (Bowden et al., 2001) which have been isolated from pigs, and Tioman virus (Chua et al., 2001) and Mapuera virus (Henderson et al., 1995; Wang et al., 2007), which have been isolated from fruit bats. More recently both fruit- and insect-eating bats have been identified as a reservoir for a number of novel rubulaviruses, including a bat rubulavirus which showed ~ 90 % sequence similarity to mumps virus (Drexler et al., 2012).

1.3 Mumps virus structure and genome

MuV particles take on many forms and range in size from 100 – 600 nm. They consist of a helical ribonucleoprotein (RNP) core containing the RNA genome. This is associated with the nucleoprotein, phosphoprotein and polymerase encoded for by the large protein. The RNP complex is surrounded by the viral matrix protein and a lipid envelope which is derived from the host cell. The small hydrophobic protein is membrane-associated and incorporated into the lipid envelope (Takeuchi et al., 1996), which is also studded with the fusion and haemagglutinin-neuraminidase viral glycoproteins, projecting 12 – 15 nm from the surface of the virion (Carbone and Rubin, 2007; Lamb et al., 2006) as depicted in Figure 1.

MuV has a non-segmented negative-sense RNA genome which consists of 15,384 nucleotides. It contains seven genes encoding for nine proteins; nucleoprotein (N), V, I, phosphoprotein (P), matrix (M), fusion (F), small hydrophobic (SH), haemagglutinin-neuraminidase (HN) and the large (L) polymerase proteins. The genome is ordered 3'–N-V/P/I-M-F-SH-HN-L-5' and each gene is separated by non-transcribed sequences as depicted in Figure 2.

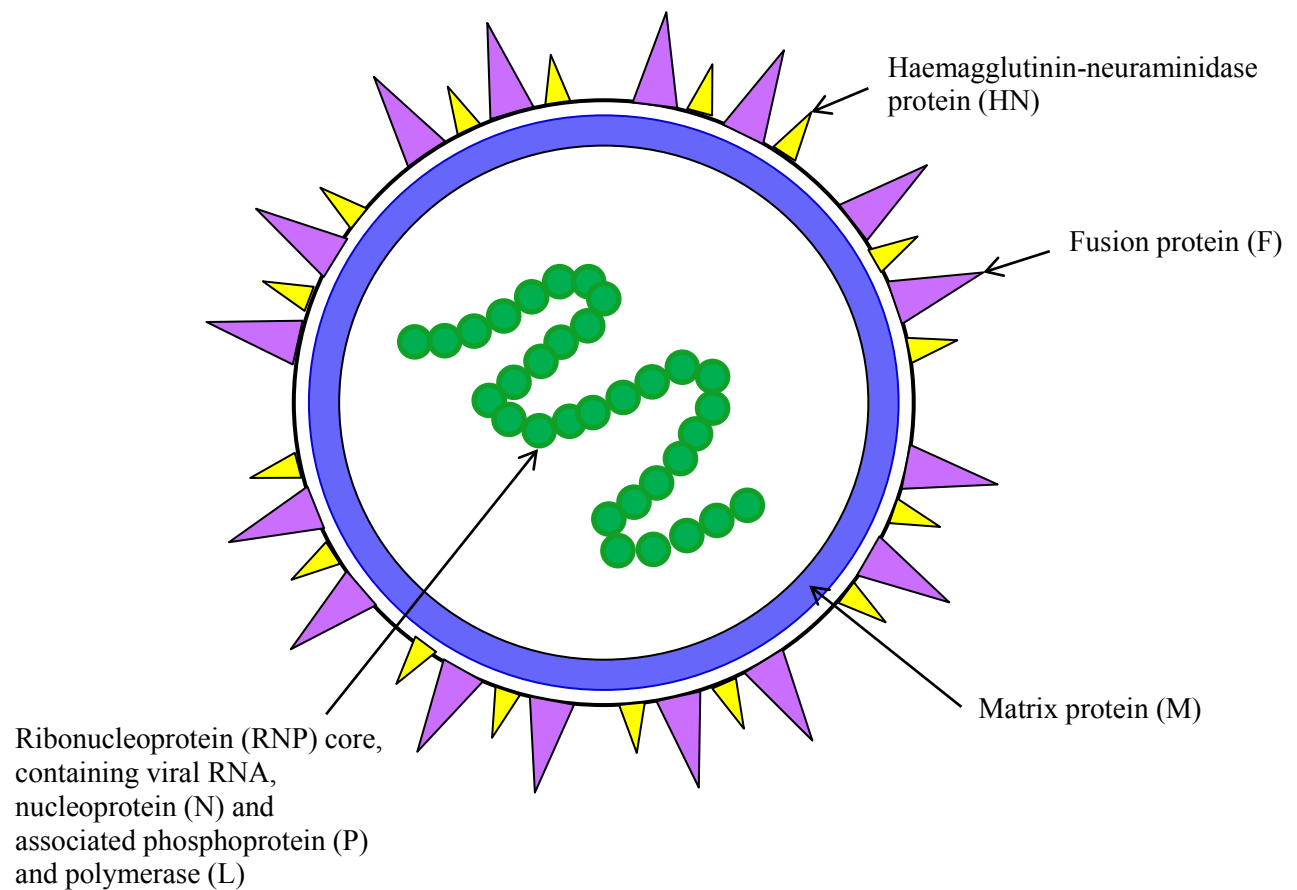


Figure 1: Schematic representation of a mumps virion.

The virion contains the helical ribonucleoprotein complex (green) of viral RNA, N, P and L proteins, surrounded by the structural M protein (blue), with the F (purple) and HN (yellow) glycoproteins protruding from the surface of the virion. The SH protein is non-structural and is not depicted.

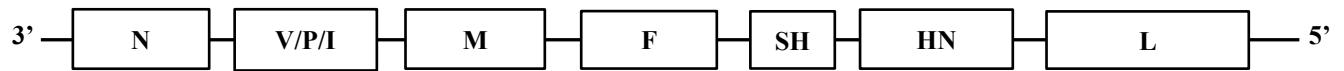


Figure 2: Schematic representation of the MuV genome organisation.

The genome is ordered from 3' to 5', with genes encoding the nucleoprotein (N), V/phosphoprotein/I, matrix (M), fusion (F), small hydrophobic (SH), haemagglutinin-neuraminidase (HN) and large (L) proteins.

1.4 Mumps virus proteins

Each of the nine MuV proteins play a vital role in the lifecycle of the virus.

1.4.1 Nucleoprotein

The nucleoprotein, has a molecular weight of approximately 62 kilo Daltons (kDa) and consists of 549 amino acids (Cox et al., 2009). It is the first protein to be translated in the viral genome and it functions as a RNA-binding protein coating the viral RNA and protecting it from nuclease digestion (Lamb and Parks, 2007). The amino-terminal domain of the nucleoprotein is highly conserved and it is this region which is responsible for RNA binding and the formation of the helical nucleocapsid (Kingston et al., 2004; Kingston et al., 2008). The carboxy-terminal domain is more variable, this is common to other paramyxoviruses, such as measles virus (Jensen et al.,

2011) and Sendai virus (Jensen et al., 2008). The carboxy-terminus of the measles nucleoprotein has been shown to alter the dynamics and structure of the nucleocapsid, with the full-length nucleocapsid appearing to be a flexible structure, which has been shown to become more rigid when the carboxy-terminal domain of the nucleoprotein is removed (Jensen et al., 2011).

The MuV nucleoprotein carboxy-terminus binds the RNA-dependent-RNA-polymerase, a complex of the P and L proteins, in a similar manner to other paramyxoviruses such as measles virus (Jensen et al., 2011), Hendra virus (Communie et al., 2013) and Sendai virus (Buchholz et al., 1994). Recently, the structure of the MuV nucleocapsid has been solved, leading to the observation that the MuV nucleocapsid binds to both the amino- and carboxy-terminal domains of the phosphoprotein (P), which has not been reported for other paramyxoviruses (Cox et al., 2014). The binding of the nucleocapsid to the amino-terminal domain of P reportedly induces uncoiling of the helical nucleocapsid, and binding to the carboxy-terminal domain of P is thought to assist the viral RNA-dependent-RNA-polymerase in gaining access to the viral RNA within the nucleocapsid allowing initiation of viral RNA synthesis.

1.4.2 V/P/I proteins

RNA editing results in the transcription of three mRNAs from the V/P/I gene, each encoding for a different protein (Paterson and Lamb, 1990). The three proteins contain the same amino-termini but differ at the carboxy-termini. The unedited transcript encodes the V protein, the addition of two guanine (g) residues produces the P protein (Elliott et al., 1990) and the addition of four g residues results in the I protein (Paterson and Lamb, 1990), as depicted in Figure 3. The P protein is the largest, comprising 391 amino acids with a molecular weight of 41 - 47 kDa

(Takeuchi et al., 1988). The V protein is 224 amino acids in length with a molecular weight of 25 - 28 kDa (Takeuchi et al., 1990) and the I protein consists of 161 amino acids and weighs approximately 19 kDa (Carbone and Rubin, 2007).

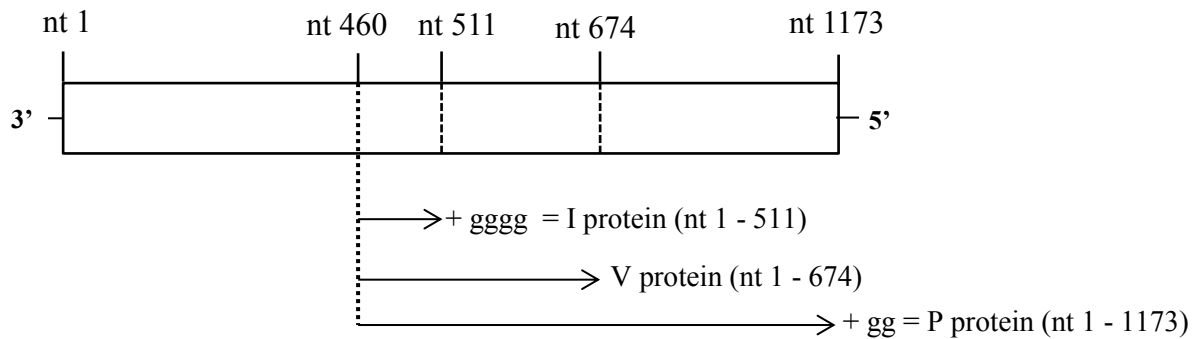


Figure 3: Schematic representation of the V/P/I gene as transcribed during RNA editing.

The addition of two or four guanine residues in the g-rich region (nt 460) produces the P and I proteins, the V protein is produced from the unedited transcript. Each protein has the same starting nucleotide (nt 1) but differing termini (nt 511 / I), (nt 674 / V) and (nt 1173 / P).

The V protein plays an essential role in the avoidance of the host cell interferon response and has been demonstrated to disrupt the signal transducer and activator of transcription-1 (STAT-1) (Kubota et al., 2001, 2002) and also STAT-3 (Puri et al., 2009; Ulane et al., 2003). It has been shown to inhibit interferon production through its interaction with melanoma differentiation-

associated gene-5 (mda-5) (Andrejeva et al., 2004), RACK1 (Kubota et al., 2002) and also to inhibit interleukin-6 signalling as a result of STAT-3 degradation (Xu et al., 2012).

The P protein is essential for viral RNA synthesis allowing interaction of the viral RNA-dependent RNA polymerase with the viral nucleocapsid (Kingston et al., 2004). The carboxy-terminus contains the nucleocapsid-binding domain at amino acids 343 – 391 (Kingston et al., 2008). It has also been reported that the amino-terminus (residues 1 – 194) of the P protein can bind nucleocapsid-like particles (Cox et al., 2013). These studies have indicated that the carboxy-terminus of the P protein interacts with the amino-terminus of the nucleocapsid and the amino-terminus of P interacts with other regions of the nucleocapsid. Recent studies (Cox et al., 2014) have suggested that the amino- and carboxy-termini of the P protein act together to allow viral RNA synthesis. The carboxy-terminus interacts with the nucleocapsid to enable recognition by the RNA-dependent RNA polymerase and the amino-terminus uncoils the nucleocapsid to allow access to the viral RNA.

The role of the I protein in *Rubulaviruses* has not been identified and studies investigating the function of the I protein are lacking.

1.4.3 Matrix protein

The matrix (M) gene encodes the structural matrix protein which is 375 amino acids in length with a molecular weight in the range of 41 – 47 kDa (Elliott et al., 1989). The M protein links the MuV nucleocapsid to the membrane of the virus particle. Studies on related paramyxoviruses such as Sendai virus (Stricker et al., 1994) and Newcastle disease virus (Pantua et al., 2006) infer that the MuV M protein interacts with the cytoplasmic tails of the fusion and haemagglutinin-neuraminidase glycoproteins, creating a bridge to the nucleocapsid at the host

cell plasma membrane (Pei et al., 2011). The M protein plays a critical role in virus assembly, with efficient production of virus-like particle (VLP) formation occurring when the M protein is co-expressed together with other viral proteins, nucleoprotein, P protein, fusion (F) protein and large (L) protein (Li et al., 2009). The assembly of the M protein at the plasma membrane of infected cells, signals for other viral components to gather at the same location to enable budding to occur (Li et al., 2009).

The role of the MuV M protein in transcriptional regulation is currently unknown. However, the M protein in other negative-strand RNA viruses; vesicular stomatitis virus (Clinton et al., 1978), influenza virus (Watanabe et al., 1996), rabies virus (Finke et al., 2003) and more recently measles virus (Iwasaki et al., 2009) have been shown play a role in inhibiting transcription and inducing genome replication.

1.4.4 Fusion protein

The fusion (F) protein is one of the surface glycoproteins which protrude from the lipid envelope. It is synthesised as an inactive precursor F_0 , which is 538 amino acids long with a molecular weight range of 64 – 74 kDa (Elliott et al., 1989). The F_0 precursor is cleaved by the proteolytic enzyme furin, at the R-R-H-K-R motif (Hosaka et al., 1991) to produce two biologically active peptides which remain bonded together by disulphide bonds (Scheid and Choppin, 1977; Server et al., 1985). The smaller amino-terminal peptide F_2 is 102 amino acids long with a molecular weight range of 10 - 16 kDa and the larger carboxy-terminal peptide F_1 , is 436 amino acids long with a molecular weight range of 56 – 61 kDa (Merz et al., 1983). The F_1 peptide contains a highly conserved, hydrophobic domain designated the fusion peptide and is thought to participate in membrane fusion (Liu et al., 2006). The cleavage of F_0 into F_1 and F_2 is

essential for virus-to-cell and cell-to-cell fusion, which is required for virus infectivity and spread (Morrison, 2003).

MuVs vary in their ability to induce fusion in cell culture, which may be strain or cell line specific. The Hoshino vaccine strain of mumps does not induce fusion in B95a cells. It was shown by co-expression of the Hoshino MuV F and HN proteins that a leucine residue (L) at position 383 was required for fusion in B95a cells (Yoshida and Nakayama, 2010). In other studies, expression of recombinant cDNA clones has been used to identify that increased fusogenicity in COS-7 cells requires a serine (S) at position 195 (Tanabayashi et al., 1993). In addition, recombinant MuVs have shown that an alanine residue (A) at position 91 produces a greater fusogenicity effect in Vero cells (Malik et al., 2007a).

It has however been identified that the F protein alone is not sufficient to induce fusion activity (Tsurudome et al., 1986). There is also the requirement for the expression of the haemagglutinin-neuraminidase (HN) protein in addition to the F protein before virus-to-cell fusion is observed (Tanabayashi et al., 1992).

1.4.5 Small hydrophobic protein

The small hydrophobic (SH) gene is the smallest of the MuV genes encoding a protein 57 amino acids long with a molecular weight of 6 kDa (Elliott et al., 1989). The SH protein is hydrophobic, membrane-associated and its sequence is highly variable. It has not been detected within MuV virions (Takeuchi et al., 1996) and it is not thought to be required for replication in embryonated eggs (Takeuchi et al., 1996) or in cell culture (Afzal et al., 1990). More recently it has been reported that the SH protein targets the cellular protein ataxin-1 ubiquitin-like

interacting protein (A1Up) which suggests a role for the SH protein in the prevention of cell apoptosis (Woznik et al., 2010).

Due to the high variability in the nucleotide sequence of the SH gene (Afzal et al., 1997), it is most commonly used for genotyping MuV strains for epidemiological purposes.

1.4.6 Haemagglutinin-neuraminidase protein

The second glycoprotein on the lipid envelope is the haemagglutinin-neuraminidase (HN) protein. It is 582 amino acids long with a molecular weight of approximately 74 – 80 kDa (Waxham et al., 1988). It is thought to be bound to the F protein (Connaris et al., 2002) until it finds and binds to its cellular receptor initiating a conformational change of the proteins and allowing the release of F (Lamb, 1993). The HN protein is responsible first for the attachment of released virus to neighbouring cells by the cell-surface receptor sialic acid and secondly for the neuraminidase activity which promotes cleavage of the sialic acid from the viral and cell surface (Malik et al., 2007b). The HN protein works together with the F protein to mediate virus-to-cell and cell-to-cell fusion enabling viral spread.

The HN protein is the major antigenic protein and is the cell surface target for neutralising antibodies which are thought to be important for protective immune response (Cusi et al., 2001; Houard et al., 1995). Therefore the HN protein has been used to determine antigenic differences between mumps strains (Yates et al., 1996). Much of what is known about mumps HN structure is inferred from other paramyxoviruses, in particular Newcastle disease virus and parainfluenza virus (Kulkarni-Kale et al., 2007). However, based on HN sequence alignments of various MuV strains and isolates, there have been nine potential N-linked glycosylation sites identified at positions; 12, 127, 284, 329, 400, 448, 464, 507 and 514, recognised by the marker sequences N-

X-S or N-X-T (Apweiler et al., 1999). These positions were found to be conserved in wild-type and vaccine MuV strains (Kulkarni-Kale et al., 2007). The amino acid regions aa265-288, aa329-340 and aa352-360 have also been proposed to be antigenic (Cusi et al., 2001; Kovamees et al., 1990; Orvell et al., 1997), with region aa329-340 reported to have shown the ability to induce neutralising antibodies to attenuated MuV and wild-type strains (Cusi et al., 2001).

1.4.7 Large protein

As its name suggests the large (L) protein is the largest of the MuV proteins. It is 2261 amino acids in length with a molecular weight of 180 – 200 kDa (Okazaki et al., 1992). Through studies from related viruses, Sendai and Newcastle disease viruses, the L protein of MuV is understood to be required for transcription and replication (Hamaguchi et al., 1983; Horikami et al., 1992). It contains the catalytic subunit conferring polymerase activity for the synthesis of viral mRNA (Kingston et al., 2004) and is the major component of the RNA-dependent RNA polymerase in complex with the P protein. This complex associates with the nucleoprotein and viral RNA to form the MuV nucleocapsid.

1.5 Pathogenesis and disease

Humans are the only natural host of MuV. Whilst there have been experimental infections of MuV in animals, the results have been inconclusive and an animal model which mimics human disease has yet to be identified (Houard et al., 1995; Johnson and Goodpasture, 1934; Parker et al., 2013; Tsurudome et al., 1987).

Mumps is commonly a childhood disease and is characterised by fever with pain and swelling of the parotid gland(s) which can be unilateral or bilateral. This is the hallmark symptom occurring

in 95 % of symptomatic patients, however one third of patients are asymptomatic (Philip et al., 1959). Whilst usually a self-limiting mild illness, some complications such as; orchitis, oophoritis, aseptic meningitis, pancreatitis and deafness can occur. MuV infection in adults can be more severe, with a higher rate of complications observed, for example orchitis and oophoritis (Gupta et al., 2005).

MuV is transmitted by aerosol droplet and direct contact with infected persons. It has an incubation period of 15 – 24 days, with the patient contagious from one to two days before the onset of clinical symptoms until around nine days after (Hviid et al., 2008). The cellular receptor for MuV is sialic acid. Sialic acid is expressed on the surface of most cells, which therefore allows MuV to infect almost all cell types. MuV replicates in the nasal and upper respiratory tract and is frequently accompanied by a transient primary viremia (Hviid et al., 2008). It is thought that the viremia is cell-associated, likely through T lymphocytes although this has not been confirmed (Fleischer and Kreth, 1982; Wolinsky et al., 1976). This may allow the virus to spread throughout other organs, in particular parotid glands but also pancreas, heart, kidneys and testes (Gupta et al., 2005; Hviid et al., 2008). In some instances infection can remain in the respiratory tract (Foy et al., 1971). MuV can also enter the central nervous system (CNS), with MuV infection the leading cause of aseptic meningitis and deafness prior to widespread vaccination coverage (Hviid et al., 2008; Rubin et al., 1998).

1.6 Diagnosis

Mumps disease is diagnosed clinically by pain and swelling of the parotid gland(s) either unilaterally or bilaterally and lasting for two or more days. As mentioned earlier, other symptoms can also present, such as orchitis, oophoritis, aseptic meningitis, pancreatitis and

deafness. Parotid gland pain and swelling can progress for around three days and lasts around one week (Hviid et al., 2008). Mumps is a notifiable disease in the United Kingdom under the Public Health (Control of Disease) Act 1984 and Health Protection (Notification) Regulations 2010. Health professionals are required to collect samples from patients presenting with suspected mumps infection, for confirmatory testing at diagnostic laboratories.

Routine laboratory diagnosis is based on the isolation of MuV, detection of viral nucleic acid or detection of mumps IgM antibody by serological methods. There is a short timeframe in which to isolate virus successfully, usually within the first week of displaying symptoms. MuV is present in saliva for up to five days after the onset of clinical symptoms (Chiba et al., 1976). Isolation of MuV from clinical samples is performed on Vero (African Green monkey kidney) cells. These are an interferon-deficient cell line, sensitive to MuV infection and are the most routinely used cell line for MuV isolation and propagation (Knowles and Cohen, 2001). Mumps-specific IgM or IgG antibodies are measured from saliva or sera, using the enzyme-linked immunosorbant assay (ELISA) method (Nicolai-Scholten et al., 1980; Ramsay et al., 1991; Ukkonen et al., 1981). In the event of an inconclusive or negative result, reverse-transcriptase polymerase chain reaction (RT-PCR) is carried out on clinical samples to identify the presence or absence of MuV nucleic acid. The SH gene is routinely amplified, which is the most variable MuV gene (Afzal et al., 1997) and also enables the clinical sample to be genotyped for epidemiological surveillance (Royuela et al., 2011).

1.7 Epidemiology

MuV is endemic throughout the world. In the absence of vaccination, mumps cases peak every two to five years, usually in the winter and spring months. Annual incidences of mumps

infections range from 100 to 1000 per 100 000 population (WHO, 2007). There are 12 recognised mumps genotypes which are designated A – N, with the exception of E and M which are now classified as genotypes C and K respectively (WHO, 2012). MuV genotype is assigned based on the sequence diversity of the small hydrophobic (SH) gene and haemagglutinin-neuraminidase (HN) gene. MuV is known to be serologically monotypic (Plotkin, 2008) and cross-neutralisation between genetically distinct mumps strains is observed. Circulating wild type MuVs are commonly genotypes C, G, H, J and K in the western hemisphere and genotypes B, F, I and L in Asia (Jin et al., 2014; Muhlemann, 2004).

Mumps is historically considered to be a childhood disease affecting children between the ages of 5 and 9 years old. However, over the past 20 years there has been a shift with the infection now typically observed in young adults aged 15 – 24 who are in university (Aasheim et al., 2014; Brockhoff et al., 2010; Centers for Disease and Prevention, 2012) or military settings (Arday et al., 1989; Eick et al., 2008), residing in close proximity and are likely to be the cohort who did not receive one or two doses of mumps-containing vaccine.

Since the introduction of mumps vaccination in the 1960s there has been a dramatic reduction in the number of cases of mumps disease to < 1 case per 100 000 population (WHO, 2007). However, in more recent years there have been large outbreaks occurring within highly vaccinated populations. The majority of these cases occur in 18 – 24 year old individuals, in the United States (Centers for Disease and Prevention, 2006) and similar trends were also observed in the United Kingdom in 2004 – 2005 (Savage, 2006), the Netherlands in 2004 (Brockhoff et al., 2010), Czech Republic 2005 – 2006 (Boxall et al., 2008) and Germany in 2010 (Otto et al., 2010) amongst others. In these outbreaks, where isolates were obtained and analysed, genotype G was common to all, which is phylogenetically distinct to genotype A (Jeryl Lynn) or genotype

B (Urabe) MuVs, which were the vaccine strains used in these countries. This highlights the possibility of waning immunity as a cause for the increase in susceptibility to mumps infection, or that some circulating wild type MuV contain antigenic differences to vaccine strains which may result in the immune escape of MuVs (Crowley and Afzal, 2002; Nojd et al., 2001).

1.8 Mumps vaccines

The first mumps vaccine was developed by Karl Habel in 1946 (Habel, 1946). It was based on a formalin-inactivated virus and was used between 1950 and 1978 in the United States. Immunity was shown to be short-lived and the vaccine was discontinued (Habel, 1951; Ukkonen and Penttinen, 1981). The advent of live attenuated mumps vaccines that were produced in embryonated hens eggs (Enders et al., 1946; Habel, 1945) and cell culture systems (Henle and Deinhardt, 1955) showed that MuV was capable of adapting to various cell substrates, including chicken embryo fibroblasts (CEFs) (Beck et al., 1989), canine kidney cells (Fedova et al., 1987), guinea-pig kidney primary cells (Odisseev and Gacheva, 1994) and human diploid cells (Gluck et al., 1986; Sassani et al., 1991). It is this adaptation to cell substrates which forms the basis of MuV attenuation for vaccine production, although a universal genetic marker for attenuation of MuV has not been determined to date (Santak et al., 2014).

The first live attenuated mumps vaccine originated from a throat culture isolate taken from the daughter of Maurice Hilleman, a scientist at Merck & Co who was responsible for vaccine development. The isolate was serially passaged through embryonated hens' eggs and chicken embryo cell culture to achieve attenuation (Buynak and Hilleman, 1966), producing the Jeryl Lynn mumps vaccine virus. This Jeryl Lynn vaccine was manufactured by Merck & Co in the

1960s and gave approximately 95 % seroconversion in recipients (Dayan and Rubin, 2008). It was later shown to be a mixture of two distinct but highly related MuV strains, Jeryl Lynn-2 (JL-2) and Jeryl Lynn-5 (JL-5) (Afzal et al., 1993; Chambers et al., 2009). The Merck & Co Jeryl Lynn MuV vaccine strain has been marketed as MumpsVax; single mumps vaccine, M-M-Vax; Measles, Mumps bivalent vaccine, MMR-II/MMRVaxPro; Measles, Mumps, Rubella (MMR) trivalent vaccine or ProQuad; MMR+Varicella (MMRV) tetravalent vaccine. The single and bivalent vaccines were discontinued in 2009 (Plotkin, 2008).

The JL-5 strain was isolated from the Merck & Co vaccine in 1998 by GlaxoSmithKline (GSK), and further passaged in chicken embryo fibroblasts (Tillieux et al., 2009). This Jeryl Lynn strain was named RIT 4385 and was shown to have a similar safety profile and efficacy as the Merck & Co Jeryl Lynn vaccine (Tillieux et al., 2009; Usonis et al., 1998). The RIT 4385 vaccine strain is marketed as Priorix; MMR trivalent vaccine or Priorix-Tetra; MMRV tetravalent vaccine.

The Merck and GSK Jeryl Lynn strains are the most widely used MuV vaccine strain (Betakova et al., 2013; Carbone and Rubin, 2007). However, there have been many other live attenuated MuV vaccines produced using different MuV strains and used worldwide, some of the more recent MuV strains used in vaccines are shown in Table 1.

Sevapharma in the Czech Republic, also manufactured a Jeryl Lynn strain mumps vaccine (Plesnik et al., 1984). For the purposes of this thesis, this Jeryl Lynn strain will be referred to as Jeryl Lynn Canine Kidney (JL-CK). The JL-CK vaccine virus was produced by passage in primary canine kidney cells (Fedova et al., 1987). The exact history of the production of the JL-CK vaccine virus is not known, however it is thought that it was produced by limiting dilution of the Merck & Co Jeryl Lynn vaccine, followed by passage in primary canine kidney cells. The JL-CK vaccine is marketed as Pavivac; monovalent mumps vaccine, Mopavac; Measles, Mumps

bivalent vaccine or Trivivac; MMR trivalent vaccine (Kubinyiova et al., 2008; Stepanova et al., 2006).

1.9 Safety, adverse events and vaccine efficacy

Immunogenicity studies on mumps vaccine strains used in clinical trials before licencing have provided estimates of efficacy based on seroconversion rates. This data combined with safety data obtained during clinical trials are used by manufacturers to apply for a medicine licence, otherwise known as a marketing authorisation. The licencing authority in the United Kingdom is the Medicines and Healthcare products Regulatory Agency (MHRA). Medicines in the UK have been regulated since the 1960s under the terms of the Medicines Act of 1968 which provides a legal framework for the control of medicines and requires medicines to be licenced before being allowed onto the UK market. More recently many of the conditions of the Act have been superseded by regulations covering the European legislation of medicines. The MHRA collaborates with the European Medicines Agency (EMA), the European regulator and the regulatory agency in the United States the Food and Drug Administration (FDA).

Once licenced, medicines are monitored for up to two years to enable reporting of additional side effects and adverse events which may not have been observed in the pre-licencing clinical trials.

Table 1: Live attenuated mumps vaccines in recent use

Strain	Genotype	Manufacturer	Substrate	Distribution	Reference
Jeryl Lynn	A	Merck & Co	CEF	Worldwide	(Buynak and Hilleman, 1966)
RIT 4385*	A	GlaxoSmithKline	CEF	Worldwide	(Tillieux et al., 2009)
JL-CK*	A	Sevapharma	Primary canine kidney	Czech Republic	(Plesnik et al., 1984)
S79*	A	Shanghai, Beijing, and Lanzhou Institute of Biological Products, China	CEF	China	(Fu et al., 2008)
Urabe AM9	B	Sanofi Pasteur, GlaxoSmithKline, Biken	CEF	Worldwide [#]	(Yamanishi et al., 1970)
Leningrad-Zagreb	N	Institute of Immunology Zagreb, Serum Institute of India	CEF	Worldwide	(Beck et al., 1989)
Leningrad-3	N	Bacterial Medicine Institute Moscow	Primary guinea-pig & Japanese quail embryo fibroblasts	Russia	(Smorodintsev et al., 1965)
Rubini	A	Swiss Serum Institute	Human Diploid	Europe [#]	(Gluck et al., 1986)
Hoshino	B	Kitasato Institute	CEF	Japan	(Makino et al., 1976)
S-12	H	Razi State Serum and Vaccine Institute	Human Diploid	Iran	(Sassani et al., 1991)

* derived from original Jeryl Lynn vaccine strain, [#] withdrawn from Europe, USA and Canada

An Adverse Event Following Vaccination (AEFI) is defined by the Council for International Organisations of Medical Science and by the World Health Organisation as “any untoward medical occurrence which follows immunisation and which does not necessarily have a causal relationship with the usage of the vaccine” (http://whqlibdoc.who.int/publications/2012/9789290360834_eng.pdf).

Different vaccine strains vary in protective efficacy and degree of attenuation (Goh, 1999), ability to cause aseptic meningitis and adverse event profiles (Brown and Wright, 1998; da Cunha et al., 2002; Gilliland et al., 2013; Kaic et al., 2008).

1.9.1 Urabe vaccine strain

The Urabe Am9 strain was developed in Japan from an isolate obtained from a mumps patient (Yamanishi et al., 1970) and attenuated by serial passage in chicken embryo fibroblasts. The Urabe-containing vaccine induced seroconversion in approximately 85 % - 94 % of recipients (Ehregut et al., 1983; Pons et al., 2000; Schlegel et al., 1999) and has been marketed as Trimovac (Sanofi-Pasteur), Immravax (GlaxoSmithKline) and Pluserix (Biken). However, the Urabe Am9 vaccine has been associated an increased incidence of vaccine-associated aseptic meningitis (Brown and Wright, 1998; Miller et al., 2007; Miller et al., 1993; Sugiura and Yamada, 1991; Ueda et al., 1995). The vaccine was found to contain a mixture of two MuVs (Brown et al., 1996). The two MuV strains identified within the Urabe vaccine differed at amino acid 335 of the HN protein, with one variant associated with the development of aseptic meningitis in vaccinees (Brown et al., 1996). This led to the withdrawal of Urabe-containing

vaccines from use in the United Kingdom, Canada and United States amongst others (Schmitt et al., 1993; WHO, 2001).

1.9.2 Leningrad-3 and Leningrad-Zagreb vaccine strains

The Leningrad-3 (L-3) strain was developed in Russia by serial passage in guinea pig kidney cell culture and Japanese quail embryo culture. The L-3 strain is the parent virus for the Leningrad-Zagreb (L-Zagreb) strain which was developed in Croatia in 1972 by passage in chicken embryo fibroblasts (Beck et al., 1989). The L-Zagreb strain was given to the Serum Institute of India (SII), where it has been used for the manufacture of MuV vaccines (Ivancic et al., 2005) and is marketed as Tresivac (Serum Institute of India). The L-3 and L-Zagreb containing vaccines have been shown to have similar seroconversion rates compared to Urabe, approximately 90 % (Beck et al., 1989; Bhargava et al., 1995; Smorodintsev, 1960; Smorodintsev et al., 1965). However, both L-3 and L-Zagreb vaccines have been linked with vaccine-associated aseptic meningitis and mumps disease (Bakker and Mathias, 2001; Cizman et al., 1989; da Cunha et al., 2002; Gilliland et al., 2013) as well as horizontal transmission of the vaccine virus (Atrasheuskaya et al., 2006; Kaic et al., 2008; Tesovic et al., 2008). Vaccines containing the L-Zagreb strain are still in use and manufactured by the Serum Institute of India.

1.9.3 Rubini vaccine strain

The Rubini strain was developed in Switzerland by serial passage of a clinical isolate in human diploid cell culture (Gluck et al., 1986). Vaccines containing the Rubini strain were considered to have a good safety profile with lower incidences of post-vaccination fever or pain and swelling at injection site, compared to a Jeryl Lynn vaccine strain (Schwarzer et al., 1998). The

Rubini strain has not been specifically linked with AEFI. However, it has been associated with mumps outbreaks in previously vaccinated populations (Goh, 1999; Pons et al., 2000; Strohle et al., 1997). Post-vaccination studies have revealed that the Rubini vaccine gave substantially lower rates of seroconversion and effectiveness compared to individuals vaccinated with Jeryl Lynn strains (Goh, 1999; Schlegel et al., 1999) with seroconversion reported to be as low as 38 % in vaccine recipients (Schwarzer et al., 1998). The World Health Organisation recommended that vaccines containing the Rubini strain should therefore not be used in national immunisation schedules (WHO, 2007). The Rubini vaccine was marketed as Triviraten (Swiss Serum Institute) before its use was discontinued.

1.9.4 Jeryl Lynn and RIT 4385 vaccine strains

Vaccines containing the Jeryl Lynn strain of mumps are considered to have a good safety profile in comparison to many of the other vaccine strains and have not been associated with AEFI (Czajka et al., 2009; Hviid et al., 2008; Lim et al., 2007; Nokes and Anderson, 1991). Jeryl Lynn mumps vaccines have also been shown to have similar seroconversion rates to Urabe-containing vaccines. The Merck & Co Jeryl Lynn and RIT 4385 strains, which are both licenced for use in the UK, were shown to induce seroconversion in approximately 95 % and 94 % of vaccine recipients respectively (Dayan and Rubin, 2008; Usonis et al., 1998). However, these seroconversion rates were determined during clinical trials before licencing and have been shown to be higher than the seroconversion rates observed once the vaccines were in routine use. The average effectiveness of Jeryl Lynn and Urabe-containing vaccines post-licensing, has been reported to be 77 %, with the Urabe vaccine strain seeming to offer greater protection (Hviid et al., 2008).

1.9.5 JL-CK vaccine strain

The JL-CK vaccine, which is not licenced for use in the United Kingdom, has been associated with vaccine failure (Mrazova et al., 2003; Stepanova et al., 2006). Clinical trials showed seroconversion rates in recipients to be approximately 75 %, six to eight weeks post-vaccination (Plesnik et al., 1984), increasing to 82 % after 35 months (Fedova et al., 1987; Mrazova et al., 2003). Despite high vaccination coverage with the JL-CK containing vaccine, a large mumps outbreak occurred in the Czech Republic between January 2005 and June 2006, with 5998 notified cases; 70 % of these had received two doses of Trivivac, the JL-CK-containing MMR vaccine (Boxall et al., 2008). This suggested the possibility of waning immunity (Limberkova and Lexova, 2014) or that that the vaccine may be over-attenuated (Goh, 1999). The JL-CK monovalent mumps vaccine Pavivac was also used briefly in the United Kingdom in some private clinics, where unlicensed medicines can be administered by private prescription. This was during the period when there was demand for a single mumps vaccine, until questions were raised about its manufacture, testing and storage (<http://webarchive.nationalarchives.gov.uk/20141205150130/http://www.mhra.gov.uk/home/groups/pl-p/documents/websiteresources/con2031109.pdf>).

1.10 Aims and objectives

Since the 1960's, live attenuated mumps vaccines have been produced in cell culture systems worldwide, yet the effects of the cell substrates on the genetics and biology of the resulting virus populations are poorly understood. The difference in seroconversion rates between the JL-CK vaccine virus and the RIT 4385 virus is interesting as they are descended from the same parental Jeryl Lynn vaccine strain, albeit with different production methods. The lack of stability of MuV

during growth in cell cultures is well known. Therefore adaptation of the JL-CK virus to primary canine kidney cells may have resulted in changes to the MuV, which may affect its ability to induce the same level of immunogenicity as RIT 4385.

This project was designed to test the hypothesis that the JL-CK and RIT 4385 viruses differ despite their shared origin. Using classical virology, molecular biology, *in vitro* and *in vivo* methods, the aims were to identify genotypic and phenotypic differences between the two viruses. This study will provide novel insights and contribute to our knowledge of MuV adaptation and biology.

CHAPTER 2 - MATERIALS AND METHODS

The viruses used in the study are detailed in Table 2. The details of media components, chemicals and reagents which were prepared in-house are detailed in Table 3.

Table 2: Vaccine viruses and clinical isolates

Virus	Information	Source/Reference
JL-CK	Live attenuated MuV component of Pavivac vaccine, Sevapharma, Czech Republic	Eva Vitková, Official Medicines and Control Laboratory, Prague, Czech Republic (Fedova et al., 1987; Limberkova and Lexova, 2014; Stepanova et al., 2006)
RIT 4385	Live attenuated MuV component of Priorix MMR vaccine, GSK, UK	(Tillieux et al., 2009)
Mu90	Clinical mumps isolate passaged four times on Vero cells. Originally isolated in London in 1988, designated Lo1/UK/88	(Afzal et al., 1997)
Jeryl Lynn	Live attenuated MuV component of MMR-II / MMRVaxPro MMR vaccine, Merck & Co, US. Contains two closely related mumps strains JL-5 and JL-2 (Afzal et al., 1993)	(Tillieux et al., 2009)
L3-SSW Pankow	Leningrad-3 MuV strain, further passaged in primary canine kidney cells for vaccine production. The vaccine did not pass clinical trials due to high incidence of aseptic meningitis (Tischer and Gerike, 2000)	Dr Annette Marketz, Robert Koch Institute, Berlin, Germany (Starke and Hlinak, 1974)

Table 3: Chemicals and reagents prepared in-house

Reagent	Components
Bactoagar (2 %)	Prepare 50 ml: 1 g bactoagar (Sigma-Aldrich, UK) 50 ml distilled H ₂ O
CMC (5 %)	Prepare 50 ml: 2.5 g (5% w/v) carboxymethylcellulose (Fisher Scientific, UK) 50 ml PBS A (1x)
Earles 20 x	Prepare 500 ml: 68 g NaCl (Fisher Scientific, UK) 10 g D(+) glucose (Merck, UK) 4 g KCl (Merck, UK) 2.10 g MgSO ₄ 7H ₂ O (Merck, UK) 1.90 g Na H ₂ PO ₄ 2H ₂ O (Merck, UK) 1.80 g CaCl ₂ 2H ₂ O (Merck, UK) Adjust to final volume of 500 ml with distilled H ₂ O Add 1 ml chloroform (Fisher Scientific, UK)
Lactalbumin hydrolysate (4 %)	Prepare 1 litre: 40 g lactalbumin enzymatic hydrolysate (Sigma-Aldrich, UK) 50 ml Earles 20 x Adjust to final volume of 1 litre with distilled H ₂ O
Methyl violet	Prepare 1 litre: 200 ml IMS (Tennant, UK) 5 g (0.5 % w/v) methyl violet (Merck, UK) 800 ml distilled H ₂ O
Neutral buffered formalin (10 %)	Prepare 1 litre: 100 ml formalin (Sigma-Aldrich, UK) 4 g NaH ₂ PO ₄ H ₂ O (Merck, UK) 6.5 g Na ₂ HPO ₄ anhydrous (Merck, UK) Adjust to final volume of 1 litre with distilled H ₂ O

Neutral red (0.17 %)	Prepare 100 ml: 0.17 g neutral red (Sigma-Aldrich, UK) 100 ml distilled H ₂ O
PBS A	Prepare 1 litre: 1 g NaCl (Fisher Scientific, UK) 0.25 g KCl (Merck, UK) 1.44 g Na ₂ HPO ₄ anhydrous (Merck, UK) 0.25 g KH ₂ PO ₄ (Merck, UK) Adjust to final volume of 1 litre with distilled H ₂ O pH 7.5 (adjusted with NaOH or HCl)
PBS 6-Salt	Prepare 10 litres: 80 g NaCl (Fisher Scientific, UK) 2 g KCl (Merck, UK) 1.3 g CaCl ₂ 2H ₂ O (Merck, UK) 1 g MgCl ₂ 6H ₂ O (Merck, UK) 29 g Na ₂ HPO 12H ₂ O (Merck, UK) 2 g KH ₂ PO ₄ (Merck, UK) Adjust to final volume of 10 litres with distilled H ₂ O
Sample buffer (4 x)	Prepare 50 ml: 250 mM tris HCl (Fisher Scientific, UK) 8 % SDS (Sigma-Aldrich, UK) 10 % glycerol (Sigma-Aldrich, UK) 0.04 % bromophenol blue (Sigma-Aldrich, UK) Adjust to 50 ml with distilled H ₂ O
TBE 10 x	Prepare 1 litre: 109.03 g tris base (Fisher Scientific, UK) 55.65 g boric acid (Sigma-Aldrich, UK) 9.31 g EDTA (Merck, UK) Adjust to final volume of 1 litre with distilled H ₂ O pH 7.5 (adjusted with NaOH or HCl)

TBS 10 x	Prepare 500 ml: 43.83 g NaCl (Fisher Scientific, UK) 6.06 g tris (Fisher Scientific, UK) 2 ml HCl (Merck, UK) pH 8.0 (adjusted with HCl)
Transfer buffer 10 x	Prepare 1 litre: 30 g tris base (Fisher Scientific, UK) 144.2 g glycine (Sigma-Aldrich, UK)
Tris/HEPES/SDS running buffer 10 x	Prepare 1 litre: 121 g tris base (Fisher Scientific, UK) 238 g HEPES (Sigma-Aldrich, UK) 10 g SDS (Sigma-Aldrich, UK)

2.1 Viruses

2.1.1 Preparation of virus aliquots

JL-CK vaccine virus was supplied lyophilised in two-dose aliquots. The vaccine was resuspended in 1400 µl Minimal Essential Medium (MEM) (Sigma-Aldrich, UK) and split into 100 µl aliquots before storage at -80 °C.

RIT 4385 virus was archived in 30 ml bulk liquid formulation. The bottle was defrosted with 100 µl aliquots prepared and stored at -80 °C.

Jeryl Lynn was archived in 150 ml bulk liquid formulation. The bottle was defrosted with 1 ml aliquots prepared and stored at -80 °C.

Mu90 was pre-aliquoted and archived at -80 °C.

L3-SSW Pankow was supplied lyophilised in a five-dose aliquot. The vaccine was resuspended in 500 µl sterile water and split into 50 µl aliquots before storage at -80 °C.

Table 4: Cell lines used in the study

Cell line	Origin	Source	References
Vero	African Green monkey kidney	NIBSC, ref no 880101*	(Yasumura and Kawakita, 1963)
MDCK	Canine kidney	NIBSC, ref no 821104*	(Madin and Darby, 1958)
SH-SY5Y	Human neuroblastoma	ECACC, cat no 94030304	(Biedler et al., 1978; Ross et al., 1983)

* NIBSC Ref No refers to in-house cell bank number

2.2 Routine cell culture

2.2.1 *Cell line maintenance*

Cell lines in Table 4 were routinely cultured in T75 flasks every 3 to 4 days after microscopic examination and when suitable confluence was obtained. Cell maintenance media for each cell line are described in Table 5. Confluent flasks of cells were sub-cultured by removing the maintenance medium and washing the cell sheet with PBS A. The cell sheet was washed with 5 ml 0.25 % trypsin-EDTA solution (Sigma-Aldrich, UK) then the trypsin solution was decanted and the flasks incubated at 37 °C until the cell sheet dislodged. A suitable volume of maintenance medium was added to each flask dependant on the sub-culture ratio to be prepared and the cells were resuspended before transfer of an appropriate volume of the resuspended cells to new T75 flask(s), containing 40 – 50 ml of maintenance medium. All cell culture procedures were carried out in a class II microbiological safety cabinet and cells were routinely incubated at 37 °C.

Table 5: Cell culture media used in the study

Medium	Components
Cell maintenance medium (Vero)	Minimum Essential Medium (MEM) (Sigma-Aldrich, UK) 1.5 % HEPES buffer (Sigma-Aldrich, UK) 5 % new-born calf serum (Sigma-Aldrich, UK) 4 % lactalbumin hydrolysate (in house) 2 % 200 mM L-glutamine (Sigma-Aldrich, UK) 1 % glucose (Sigma-Aldrich, UK)
Cell maintenance medium (MDCK)	MEM (Sigma-Aldrich, UK) 1.5 % HEPES (Sigma-Aldrich, UK) 5 % foetal calf serum (FCS) (Gibco, Life Technologies, UK) 1 % 200mM L-glutamine (Sigma-Aldrich, UK)
Cell maintenance medium (SH-SY5Y)	1:1 MEM : Ham's F12 (Sigma-Aldrich, UK) 15 % FCS (Gibco, Life Technologies, UK)
Infection medium	MEM (Sigma-Aldrich, UK) 4 % FCS (Gibco, Life Technologies, UK) 2 % 200 mM L-glutamine (Sigma-Aldrich, UK) 1 % 10,000 units/ml penicillin/streptomycin (Sigma-Aldrich, UK) 1 % 250 mM amphotericin B (Sigma-Aldrich, UK)
CMC overlay	MEM (Sigma-Aldrich, UK) 4 % FCS (Gibco, Life Technologies, UK) 2 % 200 mM L-glutamine (Sigma-Aldrich, UK) 1 % 10,000 units/ml penicillin/streptomycin (Sigma-Aldrich, UK) 1 % 250 mM amphotericin B (Sigma-Aldrich, UK) 50 ml 5 % CMC (in house)
Agar overlay	40 ml MEM (Sigma-Aldrich, UK) 50 ml 2 % bactoagar 10 ml 0.17 % neutral red

2.3 *In vitro* growth of mumps viruses

2.3.1 *Virus infection of Vero cells*

Vero cells were seeded into 24-well plates or T25 flask(s) (both Corning Inc., US) at a density of *ca.* 1×10^5 cells per well in 1 ml infection medium per well, or 5 ml infection medium per flask. Virus stocks were removed from -80°C and allowed to thaw on ice. A dilution range of each virus was prepared so that the same plaque forming units (pfu) per ml were inoculated, at approximately a multiplicity of infection (MOI) of 0.0001 pfu per cell. Wells were inoculated in triplicate with 50 μl per well or 250 μl per T25 flask of the appropriate virus dilution and incubated at 35°C , 5 % CO_2 for *ca.* 3 hr. After incubation the inoculate was removed and replaced with either 1 ml per well or 5 ml per flask of infection medium before incubating at 35°C , 5 % CO_2 for the appropriate time period for each assay, between 1 and 10 days. Plates or flasks were then removed and stored at -80°C , before titration of the viruses on Vero cells.

2.3.2 *Infection of cells and measurement of cell lysis*

Vero cells were seeded into 96-well plates (Corning Inc., US) at a density of *ca.* 2×10^4 cells per well in 200 μl of infection medium. Virus stocks were removed from -80°C and allowed to thaw on ice. 10 μl of each virus (MOI = 0.0001 pfu per cell) was added to three wells of a 96-well plate and eight replicate 96-well plates were prepared. The plates were incubated at 35°C , 5 % CO_2 , with one plate removed each day for 7 days. Each plate was washed with medium before incubation with 100 μl infection medium containing 20 % CellTiter 96[®] AQueous One Solution Cell Proliferation Assay (MTS) (Promega, UK) at 37°C , 5 % CO_2 for 1 hr before analysing on a

FLUOstar OPTIMA plate reader (BMG Labtech, Germany) set at 492 nm. The 8th plate was removed on day 7 and used to titrate the viruses.

2.3.3 *Growth in Vero cells*

Vero cells were added to three wells of seven 24-well plates. Each well was inoculated with 50 µl of 1120 pfu per ml inocula consisting of either JL-CK or RIT 4385 MuVs at a MOI of 0.0001 pfu per cell, with one well containing cells only. The plates were incubated at 35 °C, 5 % CO₂, with one plate removed daily for 7 days. After incubation the plates were stored at -80 °C. After at least one day at -80 °C, the plates were thawed and serially diluted before titration on Vero cells.

2.3.4 *Growth in Vero and MDCK cells*

Vero or MDCK cells were added to T25 flasks. Each flask was inoculated with 250 µl of 1120 pfu per ml inocula consisting of either JL-CK or RIT 4385 MuV at a MOI of 0.0001 pfu per cell and incubated at 35 °C, 5 % CO₂ for 7 days. After incubation the flasks were stored at -80 °C. After at least one day at -80 °C, the flasks were thawed, contents centrifuged at 700 *xg* for 3 min and aliquoted before titration on Vero cells.

2.3.5 *Growth in Vero, MDCK and SH-SY5Y cells*

Vero, MDCK or SH-SY5Y cells were added to T25 flasks. Each flask was inoculated with 250 µl of either JL-CK or RIT 4385 MuVs at a MOI of 0.0001 pfu per cell and incubated at 35 °C, 5 % CO₂ for 72 hr. After incubation the supernatant was removed and stored at -80 °C, fresh

infection medium was added to each flask and the flasks incubated at 35 °C, 5 % CO₂. At days 1, 4 and 7 the supernatant was removed from each flask and stored at -80 °C. Fresh media was added back to each flask before freezing at -80 °C.

After at least one day at -80 °C, the flasks were thawed and contents centrifuged at 700 *xg* for 3 min and aliquoted. The frozen cell lysate supernatant was also defrosted and both were serially diluted before titration in Vero cells.

2.3.6 *Virus concentration by sucrose gradient*

Three T75 flasks of confluent Vero cells were inoculated with either JL-CK or RIT 4385 at a MOI of 0.0001 pfu per cell, with 3 ml of infection medium added and incubated at 35 °C, 5 % CO₂ for 90 min. After incubation an additional 15 ml of infection medium was added to each flask and the flasks were re-incubated at 35 °C, 5 % CO₂ until cytopathic effects (CPE) were observed, after *ca.* 3 days incubation. The flasks were then transferred to -80 °C. After at least one day at -80 °C, the flasks were thawed and the contents clarified by centrifugation at 950 *xg* for 10 min at 4 °C.

Sucrose gradients were prepared (one per flask) in Ultra-Clear Thin wall 38.5 ml tubes (Beckman Coulter, UK) by layering sucrose solutions and virus. First, 8 ml of 60 % sucrose was added, followed by 12 ml of 25 % sucrose. Then a layer of *ca.* 15 ml clarified virus supernatant was added and the tube(s) topped up with sterile PBS. Tubes were centrifuged at 47700 *xg* for 2 hr at 4 °C. The virus concentrates were pooled by removing the virus layer at the interphase and transferring them to a clean Ultra-Clear Thin wall 38.5 ml tube. Each tube was topped up with sterile PBS and subjected to centrifugation at 82500 *xg* for 90 min at 4 °C, before removing the

supernatant and allowing the pellets to air-dry. Pellets were resuspended in 60 µl sterile PBS before storing at -20 °C.

2.3.7 *Titration of viruses*

MuV were titrated by plaque assay in Vero cells. Confluent T75 flasks of Vero cells were trypsinised into 40 ml infection media with 1 ml of cells added per well, to a 24-well plate. Serial dilutions of each virus were prepared in infection medium and 50 µl of each dilution was added in triplicate to the 24-well plate containing Vero cells. Negative control wells contained cells only. The plates were incubated for *ca.* 3 hr at 35 °C, 5 % CO₂ to allow the cells to adhere. After the incubation period, the infection media was removed from the wells and replaced with 1.25 % carboxymethylcellulose (CMC) overlay. The plates were re-incubated at the same conditions, for 10 days. The cells were fixed and stained with methyl violet and the virus titres calculated as plaque forming units (pfu) per ml. Plaque sizes were measured using ImageJ (Rubin et al., 2005; Schneider et al., 2012).

For comparison of overlay media the methodology above was followed with a 1 % bactoagar overlay used as a substitute for 1.25 % CMC. After 10 days incubation, 1 ml of 1 % bactoagar containing neutral red (0.17 %) was added to each well and incubated for 4 hr at 35 °C, 5 % CO₂, followed by incubation in the dark at 4 °C overnight.

To identify temperature-sensitive viruses, the above methodology, including CMC overlay was followed, however the incubation temperatures used were 32 °C, 35 °C, 37 °C and 40 °C.

2.3.8 *Plaque isolation*

JL-CK was grown in Vero cells as in section 2.3.7 (with agar overlay). Before the fixation step, plaques were picked to 24-well plates containing confluent monolayer(s) of Vero cells and incubated at 35 °C, 5 % CO₂ until CPE was observed, before transferring the plates to -80 °C.

2.3.9 *Plaque reduction neutralisation (PRN) assays*

Serum samples and the anti-mumps IgG human serum reference 93/582 (NIBSC in-house standard), were removed from -80 °C and thawed at room temperature before heat-inactivation by incubation at 56 °C in a water bath for 30 min, to inactivate complement.

Each serum sample and the mumps serum reference (93/582) were diluted using infection medium and serial dilutions (1:2, 1:4, 1:8, 1:16, 1:32 and 1:64) were prepared. The challenge virus (RIT 4385, JL-CK or Mu90), was added to each of the dilutions. Controls contained only the virus without serum. The samples were incubated at 4 °C for 90 min. Confluent T75 flasks of Vero cells were trypsinised into 45 ml of infection media per flask and pooled.

Each of the samples was added into 24-well plates with 1 ml of trypsinised Vero cells and mixed. The plates were incubated at 35 °C, 5 % CO₂ for 3 hr. After the incubation period the medium was removed and replaced with 1 ml 1.25 % CMC overlay. The plates were re-incubated at 35 °C, 5 % CO₂ for 10 days and then stained with methyl violet. Plaque counts were used to calculate the ND₅₀ values of the sera using the Spearman-Kärber formula (Anellis and Werkowski, 1968).

2.4 Molecular biology

2.4.1 RNA extraction

RNA was extracted using Trizol[®] LS Reagent (Life Technologies, UK) following the manufacturer's recommended protocol. Briefly, three volumes of Trizol[®] LS reagent were added to one volume of sample. Additionally 2 µl of glycogen (Life Technologies, UK) and an appropriate volume of chloroform (Sigma-Aldrich, UK) were added before mixing by vortex and incubating at room temperature for 5 min. Samples were centrifuged at 18000 *xg* for 10 min at 4 °C and the top aqueous phase containing the RNA was removed to a fresh 1.5 ml nuclease-free tube. An appropriate volume of isopropanol (Sigma-Aldrich, UK) was added and samples were incubated at -20 °C for *ca.* 30 min before centrifugation at 18000 *xg* for 10 min at 4 °C to pellet the RNA. The supernatant was removed and the RNA pellet washed with ice cold 70 % ethanol before a final centrifugation at 18000 *xg* for 10 min. The supernatant was removed and the pellet allowed to air-dry, before resuspension in 10 – 30 µl diethyl pyrocarbonate (DEPC)-treated H₂O (Sigma-Aldrich, UK) and stored at -20 °C.

2.4.2 cDNA synthesis

Random hexamer cDNA was prepared from RNA using Superscript III First-Strand Synthesis Supermix (Life Technologies, UK), following the manufacturer's protocol. Briefly, 1 – 2 µl total RNA, 1 µl random hexamer and 1 µl annealing buffer were added to a nuclease-free tube, made up to 10 µl with DEPC-treated water and incubated at 65 °C for 5 min. This was transferred to ice for 5 min before the addition of 10 µl 2 x First-Strand reaction mix and 2 µl

Superscript III enzyme. This was incubated at 25 °C for 10 min, followed by 50 °C for 50 min then 85 °C for 5 min. The resulting cDNA was stored at -20 °C.

2.4.3 Real-time quantitative polymerase chain reaction

Real-time quantitative polymerase chain reaction (qPCR) was performed on RNA samples using the TaqMan[®] EZ RT-PCR kit (Life Technologies, UK). Primers and probes, described in Tables 6 and 7, were used at a working concentration of 10 µM (primers) and 5 µM (probes). All real-time qPCR reactions were performed on a 7900HT Real Time PCR System (Life Technologies, UK) with the protocol and thermal profile detailed in Table 8.

Table 6: Primers used for real-time qPCR

Primer name	Sequence 5' – 3'	Target	Source
T701	GGCAATCCAACCTCCCTTA	SH gene of JL-5 and JL-2 mumps strains	L.C. Ozoemena, NIBSC
T702	GTTCTAGCGTGACGGATCG		
JL2fw	GCTAACCCTTCTCTATCTAATCATAACT	SH gene of JL-2	This study
JL2rev	GGACTTGCCCTAACTGAGGA		

Table 7: Probes used for real-time qPCR

Probe name	Sequence 5' – 3'	Target	Source
TP703	FAM-TATTAACTATAAGACGGCGGTGCGAT-TAMRA	SH gene JL-5	L.C. Ozoemena, NIBSC
TP704	FAM-CATTAACCATAATACGGCGGTTTCGGC-TAMRA	SH gene JL-2	

Table 8: Real-time qPCR protocol and thermal cycling conditions

PCR Protocol		Thermal Cycling Parameters
TaqMan [®] EZ RT PCR		
5x TaqMan [®] EZ Buffer	10 µl	50 °C 2 min
dNTP	3 µl	58 °C 30 min
Mn ²⁺	2.5 µl	95 °C 5 min
Primer fw (10 µM)	1 µl	94 °C 20 sec } 55 °C 1 min* } 40 cycles
Primer rev (10 µM)	1 µl	
Probe (5 µM)	0.5 µl	
RT	1 µl	
AmpErase UNG	0.25 µl	
RNA	2 µl	
Water	8.75 µl	

*data collection

2.4.4 Reverse-transcription polymerase chain reaction

The JL-5 MuV sequence (accession AF201473) was used to design over-lapping primers (Table 9) covering the whole mumps genome in *ca.* 1 kb fragments. Primers were also designed against the Leningrad-3 MuV sequence (accession AY508995), detailed in Table 10. One-Step reverse transcription (RT) PCR was performed using either the Qiagen[®] One-Step RT-PCR kit or the MyTaq[™] One-Step RT-PCR kit (Bioline, UK) following the manufacturer's recommended protocols. Primers were used at a working concentration of 10 µM for both protocols and 1 – 5 µl of RNA was used as a template. RT-PCR mixes and thermal cycling parameters for both RT-PCR kits are described in Table 11. All PCR reactions were performed on an Eppendorf MasterCycler.

Table 9: PCR primers for amplification of Jeryl Lynn-5 MuV

Primer Name	Sequence 5' – 3'	Target	Primer Name	Sequence 5' – 3'	Target
Pav1fw	GGAAGTGGTGT TTTGCGATT	N gene	Pav9fw	TGGCAGGATCACAGATCAAA	M gene
Pav2rev	CCATTTGGTCCCTAGTGCAT		Pav10rev	GGTTTTCAGTTGTCGGAAT	
Pav3fw	TATGCAATGGTGGGTGACAT		Pav11fw	AGATATGTGGGGACCAACCA	
Pav4rev	ACTTGCAACTGTGCGTTTTG		Pav12rev	ACGTGCATTTGTCTGTGCTT	
Pav5fw	GGGCGAACACGTAAACACTT		Pav61fw	GCGCAAAGGCAGAAATAAAC	
Pav6rev	GCACCTTGTCACCTTTTGT		Pav62rev	CCGGTCAAATTTGCTTGATT	
Pav35fw	CTTTTGTTCTCCGGTTCAA		Pav69fw	GAGCCAGGTACACCTTCCAA	
Pav36rev	ATCTTCGCCAATTCCACACT		Pav70rev	TGTCATCAGTCTTTGCAAGTGAT	
Pav73fw	ACCAAGGGGAGAATGAATATG		Pav77fw	TTTTGCTTCGGGTAACCTGC	
Pav74rev	GTCATCTGCAAGCAAAGCAT		Pav78rev	CTTCGAAGACTGCCCCGATTA	
Pav75rev	CCCGGAAAGAAACACGATAA		Pav47fw	AAGCAACAGGTCAGGCAACT	F gene
Pav7fw	AGAGGTCCGGGTCTTTGAGT	P gene	Pav48rev	TGCCTCATTGTATTGGCAGA	
Pav37fw	GGACCAGGAGATGTGTCGTT		Pav49fw	ATGGCGCTATCCAGCTAAGA	
Pav38rev	TGCGGTTGATCTCGAATAAG		Pav50rev	AAGGATCGCTGGTACAGTGC	
Pav57fw	TCCTCAAGTCAGCAACATGC		Pav63fw	CGGTCTAATGGAGGGTCAGA	
Pav58rev	AATTCCCACATTTGCAGGAG		Pav64rev	TGACTGCATGATGGTCAGGT	
Pav59fw	CAGTGCGAACGAGATTATGG		Pav79fw	CGGTCTAATGGAGGGTCAGA	
Pav60rev	ATGCACGCTGTCACTACAGG		Pav51fw	GGCAATCCAACCTCCCTTAT	SH gene
			Pav76fw	CAGGATGAGACCGGTGATTT	

Primer Name	Sequence 5' – 3'	Target	Primer Name	Sequence 5' – 3'	Target
Pav17fw	ACTAGCTGGTGGCCGTATGA	HN gene	Pav52rev	GAGCAGCTTTTCCGATTCAG	L gene
Pav18rev	TGCCGAAGATCATATGCTTG		Pav31fw	ATCTGACGGTGCCCATCTAT	
Pav53fw	CGTCAGGGTATCCCATGTTC		Pav32rev	GCTTGGCCCTTTAGGATTTC	
Pav54rev	GATTCACCCTGGTTGTGCTT		Pav33fw	CCGAGCACAAGAAAATGGTT	
Pav55fw	TGATCCCTGGCCATTAACTC		Pav34rev	CCAAGGGGAGAAAGTAAAATCA	
Pav65fw	TTTATCCCCACTGCAACCTC		Pav39fw	GCCCCATATGGTACTCAAGG	
Pav66rev	TATTCCACTCCCCACTCCTG		Pav40rev	CCCACTCCACTCATTGTTGA	
Pav19fw	GTGGACCCGATCTCAAAGAG	L gene	Pav41fw	CGCCCAATTACAGTATGGTG	
Pav20rev	GATGGCCCCAAAGTCTCATA		Pav42rev	TCCATTCAAACAAGTGGGGTA	
Pav21fw	TATGAGACTTTGGGGCCATC		Pav43fw	GGGGGCTTAACTTTCTTTCA	
Pav22rev	GCAGGCTGCTATCTCAAACC		Pav44rev	TGAGATTAACACGAGTGGAAGC	
Pav23fw	CATGAGATGCCTGATGATGG		Pav45fw	CGCCAAGCAGATGGTAAATA	
Pav24rev	AGCCCGCTTTAATTAATCGTT		Pav46rev	TGCAATCAGGAATTGTCCTTC	
Pav25fw	GCAATTGCTGATGTGAAACG		Pav67fw	ATTCCTCAACCCCCACTTC	
Pav26rev	CATCCTGCCATGATTCCTCT		Pav68rev	GAGCCAGGTACACCTTCCAA	
Pav27fw	GAACGAAGGGTTGCATCAAT		Pav71fw	ATCAGAGCACAGCCGAAGAT	
Pav28rev	TGGGGCTATGACATCCAAAT		Pav72rev	GTGGTGATTGCAATTGCTTG	
Pav29fw	ATGGCCATAACAGGCATCTC		Pav80rev	TGGTAACTGGCCATGCAATA	
Pav30rev	CCACTTCCCTCTGCCAAGTA		Pav81fw	TAGAGGGACGGCTGAATGTT	

Table 10: PCR primers for amplification of Leningrad-3 MuV

Primer Name	Sequence 5' – 3'	Mutation Targeted
L3 1f	ACCAAGGGGAAAATGAAGATG	Non-coding leader, K36R, E108D
L3 2r	GTCATCTGCAAGCAAGGCAT	
L3 3f	CTTTTGTTCCCTCCCGTTCA	V529A, L542L, Ter550Q, L557P, N56T
L3 4r	ATCTTAGCCAATTCCACACTT	
Pav77fw	TTTGTCTTCGGGTAACCTTGC	C244R
L3 5r	GTTTTGCAGTTGTTGGAATG	
L3 6f	AAGCAACAAGTCAGGCAACT	P93S, F295S
Pav48rev	TGCCTCATTGTATTGGCAGA	
Pav55fw	TGATCCCTGGCCATTAACTC	E569K
L3 7r	TGGTAATTGGCCATGCAATA	
L3 8f	TAGAGGGACGACTGAATGTT	D311G
Pav20rev	GATGGCCCCAAAGTCTCATA	

Table 11: PCR protocols and thermal cycling parameters

PCR Protocol		Thermal Cycling Parameters		
Qiagen® One-Step RT-PCR				
5x One-Step RT mix	10 µl	50 °C	30 min	
dNTP	2 µl	95 °C	15 min	
Primer fw (10 µM)	1.5 µl	94 °C	1 min	} 35 cycles
Primer rev (10 µM)	1.5 µl	50 °C	30 sec	
RT	2 µl	72 °C	1 min	
RNA	1 - 5 µl	72 °C	10 min	
Water	28 - 32 µl	4 °C	HOLD	
Bioline MyTaq™ RT-PCR				
2x One-step mix	25µl	48 °C	20 min	
Primer fw (10 µM)	1.5 µl	95 °C	1 min	
Primer rev (10 µM)	1.5 µl	95 °C	15 sec	} 35 cycles
RT	0.5 µl	50 °C	15 sec	
RiboSafe RNase Inhibitor	1 µl	72 °C	30 sec	
RNA	1 - 5 µl	72 °C	10 min	
Water	19.5 - 23.5 µl	4 °C	HOLD	
Qiagen® HotStarTaq				
2x HotStarTaq mix	25 µl	95 °C	15 min	
Primer fw (10 µM)	1 µl	94 °C	30 sec	} 35 cycles
Primer rev (10 µM)	1 µl	50 °C	30 sec	
Water	18 µl	72 °C	1 min	
cDNA	5 µl	72 °C	10 min	
Phusion® High Fidelity mastermix				
2x Phusion mix	25 µl	98 °C	1 min	
Primer fw (10 µM)	2.5 µl	98 °C	10 sec	} 28 cycles
Primer rev (10 µM)	2.5 µl	65 °C	30 sec	
Water	18 µl	72 °C	1 min	
cDNA	2 µl	72 °C	10 min	

2.4.5 *Polymerase chain reaction (PCR)*

PCR reactions were prepared with cDNA as the template, using the Qiagen[®] HotStarTaq mastermix. The reaction mix and cycling conditions are described in Table 11, all PCR reactions were performed on an Eppendorf MasterCycler.

2.4.6 *DNA gel electrophoresis*

All PCR products were visualised on 1 % Tris-Borate-EDTA (TBE) agarose gels containing a ethidium bromide (Promega, UK) at a final concentration of 1 µg/ml, or SYBR[®] Safe (Life Technologies, UK), using a UV transilluminator (UVP Bio-Imaging Systems).

2.4.7 *PCR purification*

All PCR products were gel-purified before further downstream applications, using either the Qiagen[®] QIAquick Gel Extraction or the Bioline ISOLATE PCR and Gel kits following the manufacturer's recommended protocols. PCR products were briefly visualised using a UV transilluminator as above before excision from the agarose gel(s) using a sterile scalpel and transferred to a nuclease-free microfuge tube before weighing.

For the Qiagen[®] QIAquick Gel Extraction kit, all centrifugation steps were performed at 18000 *xg* for 1 min at room temperature. Briefly, three volumes of Buffer QG were added to one volume of gel slice and incubated in a water bath at 50 °C for 10 min or until the gel slice dissolved. One volume of isopropanol was added and mixed by vortex before transferring to a QIAquick column in a collection tube and centrifuged. The flow-through was discarded and 500 µl Buffer QG was added to the column(s), centrifuged, flow-through discarded and 750 µl Buffer

PE added to the column to wash. The flow-through was discarded and a final centrifugation step performed to ensure all Buffer PE was removed. Each column was transferred to a clean nuclease-free tube and 30 µl Buffer EB was added to the column(s) which were allowed to stand at room temperature for 1 min before centrifugation. The eluted purified PCR product(s) were stored at -20 °C.

For the Bioline ISOLATE Gel and PCR kit all centrifugation steps were performed at 10000 *xg* for 1 min at room temperature. Briefly 650 µl Gel Solubilizer was added to the gel slice and incubated in a water bath at 50 °C for 10 min or until the gel slice dissolved, 50 µl Binding Optimizer was added and mixed by vortex before transferring to a spin column in a collection tube and centrifuged. The flow-through was discarded and 750 µl Wash Buffer A added to the column(s) and centrifuged, the flow-through was discarded and the wash step repeated. The flow-through was again discarded and the spin column(s) centrifuged for 2 min to ensure complete removal of the wash buffer. Each column was transferred to a clean nuclease-free tube and 30 µl Elution Buffer was added to the column(s) which were allowed to stand at room temperature for 1 min before centrifugation at 6000 *xg*. The eluted purified PCR product(s) were stored at -20 °C.

2.4.8 Mutant analysis by PCR and restriction enzyme cleavage (MAPREC)

Mutagenic primers, described in Table 12, were designed to cover each specific mutation allowing a single unique restriction enzyme site to be introduced into only one of the PCR products (either JL-CK or RIT 4385). PCR reactions were prepared with cDNA, mutagenic primers and Qiagen® HotStarTaq mastermix, as described in section 2.4.5. PCR products were

visualised as described in section 2.4.6 and purified using QIAquick[®] PCR Purification Kit (Qiagen, UK) following the manufacturers protocol, eluting the DNA in 30 µl water.

In nuclease-free tubes, 15 µl of purified PCR product, 2 µl 10 x restriction buffer, 1 µl restriction enzyme and 2 µl water or BSA (if required) were added. The tubes were incubated in a water bath at 37 °C for 1 hr. Undigested PCR product and the digested PCR product were run on a 10 % TBE acrylamide gel for 1.5 hr at 100 V, before staining with ethidium bromide and visualised using a UV transilluminator.

2.4.9 *Sanger sequencing*

Samples were prepared by mixing 7.5 µl of purified PCR product, 7.5 µl dH₂O and 2.25 µl, 10 mM of either forward or reverse primer used in the RT-PCR, in a nuclease-free tube. The PCR product/primer mix was sent to Eurofins MWG Operon for the Value Read Tube Sequencing Service. Sequencing was performed by chain-terminating Sanger sequencing and the sequence trace files were analysed using Lasergene 8.0 – SeqMan software (DNASTAR) and aligned using ClustalW (EMBL-EBI).

Table 12: Primer sequences for MAPREC analysis

Forward primer	Sequence 5' – 3'	Reverse primer	Sequence 5' – 3'	Restriction site	Restriction Enzyme
E108Dfw	CTGTGAGATTGATGGTTT <u>GGA</u>	E108Drev	CGATCCAGGAACTGCTCAAT	GGATCC	BamHI
V529Afw	ATGCAAGGGGCGAAC <u>CC</u>	V529Arev	ATTTGGGCAGTTAGCTGTGG	CCGC	AciI
L542Lfw	CACG <u>C</u> AAACACTTTCCCAAAC	L542Lrev	CCCGGAAAGAAACACGATAA	CAGCTG*	PvuII
Ter550Qfw	GTGGGAGACTGGGAT <u>GCG</u>	Ter550Qrev	CACCGGTCTCATCCTGTTTT	GCGC	HhaI
L557Pfw	GGGGCTGGAACACCAAGATT	L557Prev	ATTGTTGCGATTGGGG <u>CT</u>	CTAG	BfaI
N56Tfw	ATTGGCAATCCAGAGCA <u>GAA</u> G	N56Trev	GATCTTCGCCAATTCCACAC	GAAGAC	BbsI

*Restriction site found within fragment, not introduced with primer

Restriction site bases integrated within primer are identified by **bold** font and the mutagenic bases are identified by underlining.

2.5 Deep sequencing

2.5.1 *Generation of long range amplicons for shotgun sequencing*

Oligo dT cDNA and gene-specific primer cDNA was synthesised as in section 2.4.2. Combinations of primers Pav1fw, Pav2rev, Pav8rev, Pav20rev, Pav25fw, Pav34rev, Pav36rev, Pav47fw, Pav48rev, Pav55fw, Pav73fw and Pav80rev, detailed in Table 9 were used to amplify 1 - 5 kb fragments of the MuV genome using Phusion[®] High-Fidelity PCR mastermix (New England Biolabs, UK), following the protocol and thermal cycling profile in Table 11, adjusting the annealing temperature to 53 °C and the volumes of cDNA to 1 µl, 2.5 µl, 3 µl or 5 µl.

2.5.2 *Amplicon-based deep sequencing*

Random hexamer cDNA was synthesised as in section 2.4.2. Fusion primers were designed to amplify *ca.* 400 bp fragments covering the regions of interest of the MuV genome, all primers contained universal tails as detailed in Table 13. The fusion primers were originally designed for use in 454 sequencing, therefore incorporate “adaptor” and “key” sequences which did not interfere with the Illumina sequencing method. The “adaptor” and “key” sequences were removed during downstream processing of the sequence reads. Amplicons were generated using Phusion[®] High-Fidelity PCR mastermix (New England Biolabs, UK) following the protocol and thermal cycling profile detailed in Table 11. The amplicons were purified to remove short fragments and primer dimers using Agencourt AMPure XP Beads (Beckman-Coulter, UK). Briefly, in nuclease-free tubes an appropriate volume of PCR amplicon(s) was added to 72 µl AMPure beads and incubated at room temperature for *ca.* 10 min. The tube(s) were placed on a Magnetic Particle Concentrator (MPC) (Life Technologies, UK) for *ca.* 5 min, then the

supernatant was removed, 200 µl 70 % ethanol was added before mixing by vortex and placing back in the MPC. The supernatant was again removed and replaced with 200 µl 70 % ethanol before mixing and replacing on the MPC. The supernatant was removed and the pellet dried at 37 °C before resuspending in 10 µl TE buffer (Sigma-Aldrich, UK). The tubes were placed in the MPC and the supernatant containing the purified amplicons was transferred to a clean nuclease-free tube and stored at -20 °C. Purified amplicons were quantified using the Agilent high sensitivity DNA kit (Agilent Technologies, US) following the manufacturer's protocol and analysing on the Agilent 2100 Bioanalyser (Agilent Technologies, US). The concentration of amplicons was determined using Qubit™ dsDNA HS Assay kit (Life Technologies, UK) following the manufacturer's protocol, with samples analysed on the Qubit® 2.0 Fluorometer (Life Technologies). Purified amplicons were pooled in equimolar concentration (2 ng/µl) and libraries prepared by Nextera® XT DNA sample preparation kit (Illumina, UK) following the manufacturer's protocol, before running on an Illumina MiSeq Sequencer.

Table 13: Fusion primers for deep sequencing

Primer	Sequence 5' – 3'	Mutation targeted
Name	ADAPTOR – <u>KEY</u> – Mumps-specific sequence	
FP1fw	CGTATCGCCTCCCTCGCGCCAT <u>TCAG</u> ACCAAGGGGAGAATGAATATG	Non-coding leader
FP1rev	CTATGCGCCTTGCCAGCCCGCT <u>TCAGG</u> CTTCGGGTGATCTATCTGC	
FP2fw	CGTATCGCCTCCCTCGCGCCAT <u>TCAGT</u> CACAGGGTAGGTGCATTGA	E108D
FP2rev	CTATGCGCCTTGCCAGCCCGCT <u>TCAGG</u> CAGCATGTATCTTGCCCTCA	
FP3fw	CGTATCGCCTCCCTCGCGCCAT <u>TCAGT</u> CCTCAAGTCAGCAACATGC	V529A, L542L, Ter550Q, L557P
FP3rev	CTATGCGCCTTGCCAGCCCGCT <u>TCAGC</u> ACCGGTCTCATCCTGTTTT	
FP4fw	CGTATCGCCTCCCTCGCGCCAT <u>TCAGT</u> CATTTCCTATCCACCCCAAT	N56T
FP4rev	CTATGCGCCTTGCCAGCCCGCT <u>TCAGC</u> CCCCCTCTTAAATTCTGTCACC	
FP5fw	CGTATCGCCTCCCTCGCGCCAT <u>TCAGC</u> TGATGGAGAAGGGTGCATT	C244R
FP5rev	CTATGCGCCTTGCCAGCCCGCT <u>TCAGG</u> AGGCAGACTTCCTGAATGG	
FP6fw	CGTATCGCCTCCCTCGCGCCAT <u>TCAG</u> ACCGAATATCCAACCCACTG	P93S
FP6rev	CTATGCGCCTTGCCAGCCCGCT <u>TCAGT</u> AATCGGTGACAATGCTGGA	
FP7fw	CGTATCGCCTCCCTCGCGCCAT <u>TCAGC</u> GGTCTAATGGAGGGTCAGA	F295S
FP7rev	CTATGCGCCTTGCCAGCCCGCT <u>TCAGT</u> GACTGCATGATGGTCAGGT	
FP8fw	CGTATCGCCTCCCTCGCGCCAT <u>TCAG</u> ACACCACAACCACCTGCTTT	E569K
FP8rev	CTATGCGCCTTGCCAGCCCGCT <u>TCAGC</u> TCTTTGAGATCGGGTCCAC	
FP9fw	CGTATCGCCTCCCTCGCGCCAT <u>TCAGT</u> GCACTACTCATGGGGGATA	D311G
FP9rev	CTATGCGCCTTGCCAGCCCGCT <u>TCAGC</u> GCATCTCAATGAGGCTTTT	

2.5.3 *Deep sequencing data analysis*

Sequencing data in the form of FASTQ files were exported into Geneious software version 6.1.3 (Biomatters Ltd). The reads were paired and the ends trimmed using the fusion primer sequences (Table 13), the Nextera transposase sequences and the Nextera index primer sequences (http://supportres.illumina.com/documents/documentation/chemistry_documentation/experiment-design/illumina-customer-sequence-letter.pdf), allowing for an 8 base-pair (bp) overlap and 0.01 error probability. Once the sequences were trimmed, the long fragments were extracted, in the range of 50 bp to 251 bp and these were mapped to the JL-5 database reference sequence (AF201473). Single nucleotide polymorphisms (SNPs) were identified by way of a nucleotide variation to the reference sequence at a minimum frequency of 0.01 % on regions where coverage was greater than 2000 reads.

2.6 Mumps protein analysis

2.6.1 *Polyacrylamide gel electrophoresis*

Concentrated and purified JL-CK and RIT 4385 viruses were prepared in triplicate for electrophoresis as follows; 20 µl virus concentrate, 10 µl 4 x sample buffer and 2 µl β-mercaptoethanol, were added to 1.5 ml tubes and denatured at 95 °C for 5 min. The samples were loaded on to 12 % Precise™ Tris-HEPES polyacrylamide gel(s) (Life Technologies, UK), with one lane containing 5 µl full-range Amersham Rainbow Marker (GE Healthcare, UK). Gel(s) were electrophoresed at 100 V constant for 2 hr. To visualise the protein bands, gel(s) were stained using the Novex Colloidal Blue staining kit (Life Technologies, UK). First, the gel(s) were fixed for 10 min in a solution of 50 % methanol and 10 % acetic acid. The fixative

was removed and the gel(s) were stained in a solution consisting of 55 ml water, 20 ml methanol and 20 ml stain A for 10 min at room temperature before 5 ml of stain B was added and incubated overnight at room temperature on a rocking platform. Finally the stain was removed and the gel(s) were placed in water until ready for analysis.

2.6.2 *Western Blotting*

Unstained polyacrylamide gel(s) containing JL-CK or RIT 4385 as in section 2.6.1 were wet blotted at room temperature, overnight with 40 mA onto PVDF membrane. The membrane was subjected to blocking for 1 hr at room temperature with TBS containing 0.1 % Tween 20 and 5 % (w/v) milk powder. A 1:10000 dilution of rabbit α -mumps nucleoprotein (a kind gift from Dr Steven Rubin, F.D.A., US) was prepared in TBS containing 0.1 % Tween 20 and 5 % (w/v) milk powder and incubated for 1 hr at room temperature on a rocking platform. The membrane was subjected to three, 10 min room temperature washes with TBS containing 0.1 % Tween 20. A 1:2000 dilution of goat α -rabbit HRP (Dako, UK) was prepared in TBS containing 0.1 % Tween 20 and 5 % (w/v) milk powder and incubated for 1 hr at room temperature on a rocking platform. The membrane was washed three times, 10 min each wash, at room temperature with TBS containing 0.1 % Tween 20. Mumps nucleoprotein was visualised using the Immobilon Western Chemiluminescent Kit (Merck Millipore, UK). Briefly, a working HRP substrate was prepared by adding 3 ml luminol reagent to 3 ml peroxide allowing the solution to incubate at room temperature for 10 min. After placing the membrane protein side up on cling film, the membrane was covered in HRP substrate and allowed to incubate at room temperature for 5 min, before draining off excess substrate. The membrane was covered in cling film and air bubbles removed before exposing the membrane to x-ray film for an appropriate exposure time.

2.6.3 Mass spectrometry

Gel bands within the molecular mass region of 52 – 76 kDa, identified by Western blot and described in section 2.6.2, were excised, washed and trypsin digested. Tryptic peptides were extracted and subjected to online nanoscale liquid chromatography coupled to tandem mass spectrometry (nano-LC/MS/MS) analysis using a LTQ-Orbitrap mass spectrometer system (Thermo Scientific, UK) (Chen et al., 2011). Mass spectra were processed and the database searched using Proteome Discoverer v.1.2 with built-in Sequest (Thermo Scientific, UK) and UniProt FASTA database release 56.4 (www.uniprot.org). A FASTA sequence file of the JL-CK nucleoprotein containing the additional amino acids at the C-termini (HANTFPNNPNQNAQLQVGDWDEQITDMIIKP/KLTPIATIPGQSSHS) was also created. Mass spectrometry was performed by Dr Jun X. Wheeler, Laboratory for Molecular Structure, NIBSC.

2.7 *In vivo* studies

2.7.1 Ferret studies

Groups of three male ferrets aged between 6 – 12 months were inoculated intra-nasally with *ca.* 2×10^4 pfu in 400 μ l (200 μ l per nostril) of either, JL-CK, RIT 4385, JL-CK1, JL-CK2, JL-CK3, JL-CK4, JL-CK5 or the “Parental” virus isolated from the JL-CK vaccine. The titres of the remainder of the inocula were checked after inoculation by titration on Vero cells.

Each study ran for 28 days, with pre-bleed samples collected 7 days before inoculation. Blood samples were collected on days 7, 14, 21 post-infection, with a terminal bleed taken on day 28.

Clinical signs including temperature, weight, parotid gland(s) and testicle swelling were measured throughout the studies.

All animal procedures were performed by fully trained and licensed NIBSC Biological Services Division staff.

2.7.2 Blood sample processing

Blood samples were collected in serum-gel clotting activator tubes (Sarstedt Ltd, UK) and allowed to clot at room temperature for 25 min. The tubes were centrifuged at 15000 $\times g$ for 5 min at 4 °C to allow separation of the serum and red blood cells. Serum was removed from the top layer of the separation gel and split into two aliquots and stored at -80 °C.

2.7.3 Rat neurovirulence

In each of the studies neonatal rat pups were inoculated intra-cerebrally within 12 – 24 hours of birth with 100 pfu in 20 μ l, adapted from (Rubin et al., 2005). The titre of each inoculum was confirmed by titration on Vero cells.

In the initial study, groups of 22 neonatal rats were inoculated either with JL-CK, RIT 4385 or Mu90 MuV's. This study ran for 25 days with three rat pups in each group sacrificed at days 1, 4, 7 and 25 with the brains harvested for virus titration. At day 25 the remaining rats were sacrificed with the brains removed and fixed in 10 % neutral-buffered formalin for determination of hydrocephalus severity.

In the second study, groups of six neonatal rat pups were inoculated as in the initial study, with either JL-CK, RIT 4385, JL-CK1, JL-CK2, JL-CK3, JL-CK4, JL-CK5 or the “Parental” virus isolated from the JL-CK vaccine.

This study ran for 25 days, the rat pups in each group were sacrificed at day 25 with the brains removed and fixed in 10 % neutral-buffered formalin for determination of hydrocephalus severity.

In the third study, groups of 16 neonatal rat pups were inoculated as in the initial study, with either JL-CK, RIT 4385, JL-CK1, JL-CK2, JL-CK3, JL-CK4, JL-CK5 or the “Parental” virus isolated from the JL-CK vaccine. This study ran for 10 days with three rat pups in each group sacrificed at days 1, 4, 7 and 10 and the brains harvested for virus titration.

2.7.4 Virus titration from rat brains

Brain weights were recorded and the required volume of cold MEM (Sigma Aldrich, UK) was added to prepare a 20 % homogenate for each brain. The brains were homogenised for 7 sec at 5000 rpm in a Precellys 24[®] cell lysis and tissue homogeniser (Bertin Technologies, France) using 2.8 mm ceramic beads (Bertin Technologies, France). 1 ml of homogenate was removed, centrifuged at 9300 $\times g$ for 5 min at 4 °C and the supernatant transferred into a clean tube. The homogenate, supernatant and pellet were all stored at -80 °C. The supernatant from each brain homogenate was serially diluted and titrated on Vero cells as described in section 2.3.7.

2.7.5 Processing of fixed brains

Fixed brains were sectioned and stained (Propath UK Limited). Briefly, the brains were cut along the anatomical midline, processed and paraffin embedded before sectioning and staining with haematoxylin and eosin as described previously (Rubin et al., 2005). Hydrocephalus severity was calculated as a percentage of the cross-sectional area of the ventricle from the area of the whole brain using ImageJ (Rubin et al., 2005; Schneider et al., 2012).

2.8 Designated accession numbers

The sequences of the five JL-CK virus variants have been deposited in GenBank and designated accession numbers as follows; JL-CK1 JQ946550, JL-CK2 JQ946551, JL-CK3 JQ946552, JL-CK4 JQ946553 and JL-CK5 JQ946554.

CHAPTER 3 – COMPARISON OF JL-CK AND RIT 4385 VACCINE VIRUSES

Mumps vaccines are live viruses which have usually been attenuated by passage in embryonated hens' eggs and/or in chicken embryo fibroblasts (CEFs). The Jeryl Lynn strain of mumps is the most commonly used strain in mumps vaccine production worldwide (Carbone and Rubin, 2007). The RIT 4385 vaccine virus is the JL-5 mumps strain which originated from the Jeryl Lynn vaccine, containing the JL-2 and JL-5 strains, and has been further propagated on CEFs. In contrast, the JL-CK vaccine virus which is also derived from the Jeryl Lynn vaccine has been further propagated on primary canine kidney cells, the use of which is atypical for mumps vaccine production. The JL-CK vaccine has been associated with vaccine failure (Mrazova et al., 2003). This may in part be related to its production. In order to identify any differences in the biological characteristics of the two viruses a number of *in vitro* and *in vivo* assays were performed.

3.1 Plaque morphology and size

Mumps virus (MuV) isolates are routinely cultured using the African Green monkey kidney (Vero) cell line. This cell line is considered to be the “gold standard” and is used in clinical laboratories to isolate and propagate MuV from clinical specimens.

Plaque assays on Vero cells were performed on both the JL-CK vaccine and RIT 4385 bulk material, in order to check viral titres and to confirm that the JL-CK vaccine contained live virus. The virus titre for JL-CK was in line with the titre given by the manufacturer. A representative plaque assay showing the plaque morphologies of both viruses is shown in Figure 4.

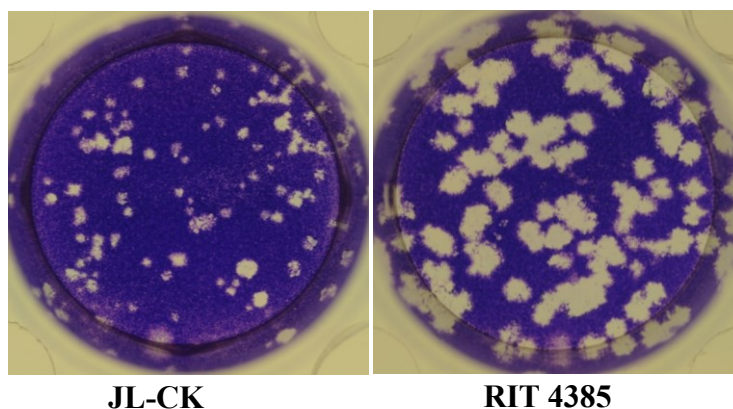


Figure 4: Plaque morphology after infection of Vero cells with either JL-CK or RIT 4385.

Vero cells were inoculated a MOI of 0.0001 pfu per cell and incubated at 35 °C, 5 % CO₂ for 10 days before fixing and staining with methyl violet to visualise plaques.

It was apparent from the plaque morphology that the JL-CK and RIT 4385 MuVs are different. JL-CK produced a mixture of small and large plaques whereas RIT 4385 formed a population of larger plaques. The plaques produced from both JL-CK and RIT 4385 viruses were measured using ImageJ software and are compared in Figure 5. A total of 25 JL-CK plaques and 33 RIT 4385 plaques were measured. The JL-CK MuV produced plaques that were at least three times smaller (by area) than those formed by RIT 4385; the analysis of the data by student's *t*-test identified the difference in plaque size between the two groups to be significantly different where $p < 0.0001$.

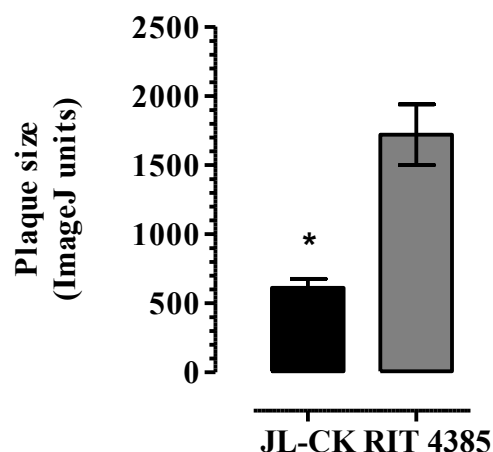


Figure 5: Plaque size produced by JL-CK and RIT 4385 in Vero cells.

Plaque areas were measured using ImageJ software. The bars represent the standard error of the mean for each group (n = 25, JL-CK; n = 33, RIT 4385). A student's *t*-test was applied to the data with a *p* value < 0.05 considered significant, * *p* < 0.0001.

It has been reported (Omata et al., 1986; Wright et al., 2000) that plaque-forming viruses, such as MuV or poliovirus can display a small plaque phenotype that is linked to temperature sensitivity and differences in overlay media have been shown to affect plaque size and clarity in influenza virus (Matrosovich et al., 2006). In order to determine whether either of these conditions might explain the small plaque phenotype observed with the JL-CK MuV, plaque assays were performed on Vero cells incubated at 32 °C, 35 °C, 37 °C or 40 °C. In each case the plaque sizes remained consistent throughout. Plaque assays were also performed comparing solid and semi-

solid overlay media. Vero cells were infected with JL-CK and RIT 4385 and overlain with either CMC (semi-solid) overlay or bactoagar (solid). Again, there was no observed difference in plaque size.

The distinct plaque size differences produced by the JL-CK vaccine were therefore not due to temperature sensitivity or overlay medium but more likely due to the presence of two or more populations of virus. The smaller plaque size may also indicate a growth defect or an issue with budding and/or fusion of the virus particles from/to neighbouring cells.

3.2 Real-time qPCR

The original Jeryl Lynn vaccine manufactured by Merck & Co was shown to be a mixture of two distinct but highly related MuV strains, JL-2 and JL-5 (Afzal et al., 1993). The major JL-5 component was isolated from the Jeryl Lynn vaccine by GlaxoSmithKline, passaged in CEFs and named RIT 4385. The JL-CK vaccine strain was also developed from the original Jeryl Lynn vaccine (Limberkova and Lexova, 2014), by passage in primary canine kidney cells, although the exact history of its production is not available. To determine whether the JL-CK vaccine is composed of JL-2 and JL-5 MuVs, a strain-specific probe-based real-time qRT-PCR assay was performed on RNA from individual JL-CK plaques. Primers and probes were designed against the highly variable SH gene. Their sequences are detailed in Tables 6 and 7. A graph showing the amplification of either JL-2 or JL-5 is depicted in Figure 6.

All plaques analysed, except the positive control (plaque 2) which contained RNA extracted from the original Jeryl Lynn vaccine (Merck & Co), showed amplification of only the JL-5 MuV sequence, not the JL-2 MuV sequence. Therefore the JL-CK vaccine contains only the JL-5 MuV.

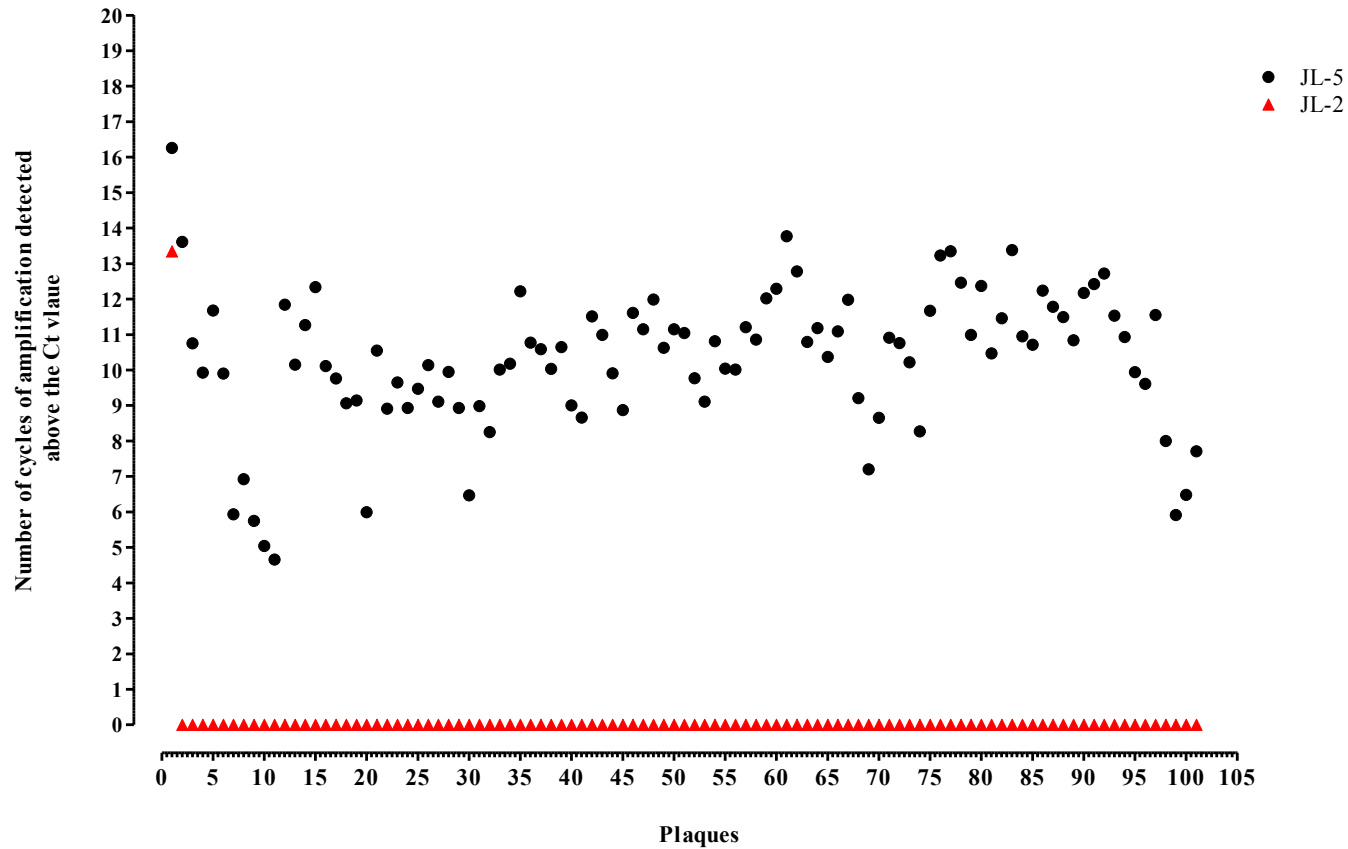


Figure 6: Real-time qPCR of individual plaques purified from JL-CK vaccine to identify the MuV strain (JL-5 or JL-2).

Each point represents one plaque, reactions were performed in duplicate. The average number of cycles of amplification detected above the Ct value is plotted. Plaque 1 is the negative control containing no RNA, plaque 2 is the positive control containing RNA from the Jeryl Lynn vaccine consisting of JL-5 and JL-2 viruses.

3.3 *In vitro* characteristics

3.3.1 *Growth of JL-CK and RIT 4385 in Vero cells*

In order to identify if there were differences in growth, Vero cells were infected with either JL-CK or RIT 4385 MuVs at a MOI of 0.0001 and incubated. At each daily timepoint the progeny viruses were titrated in Vero cells and the results calculated as the pfu per cell, Figure 7.

The data in Figure 7 show that the JL-CK virus grows as well if not slightly better than the RIT 4385 virus in Vero cells and therefore the small plaque phenotype is not due to a growth defect of the JL-CK virus.

The ability of JL-CK and RIT 4385 to lyse Vero cells was also compared. Vero cells were inoculated at the same MOI (0.0001 pfu/cell) with JL-CK or RIT 4385 MuVs and incubated. Lysis of cells was determined as a measure of cell viability using the Cell Titer 96[®] Aqueous Non-Radioactive Cell Proliferation Assay (MTS) (Promega, UK). Over the course of 7 days, cell viability was measured on JL-CK and RIT 4385 infected Vero cells and the results expressed as percentage (%) cell survival as shown in Figure 8. Both JL-CK and RIT 4385 lyse Vero cells at comparable rates, 3.7 days for JL-CK and 4 days for RIT 4385.

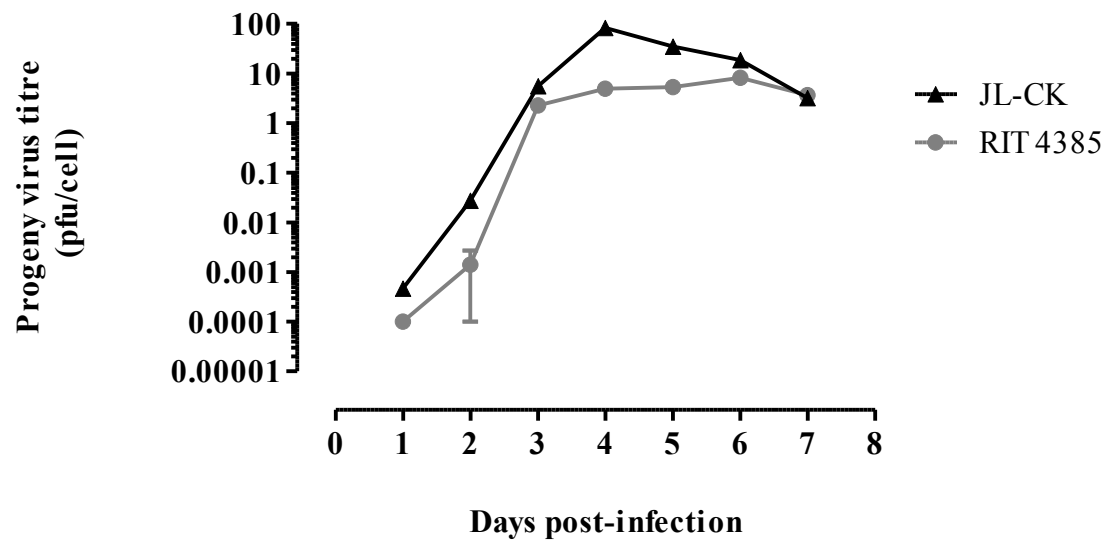


Figure 7: Progeny virus titres from JL-CK and RIT 4385 infected Vero cells.

Cells were infected at a MOI of 0.0001 pfu per cell and samples were harvested daily by freeze-thawing of the cells, followed by plaque assay in Vero cells. Each timepoint was titrated in triplicate, the error bars represent the standard error of the mean for each timepoint.

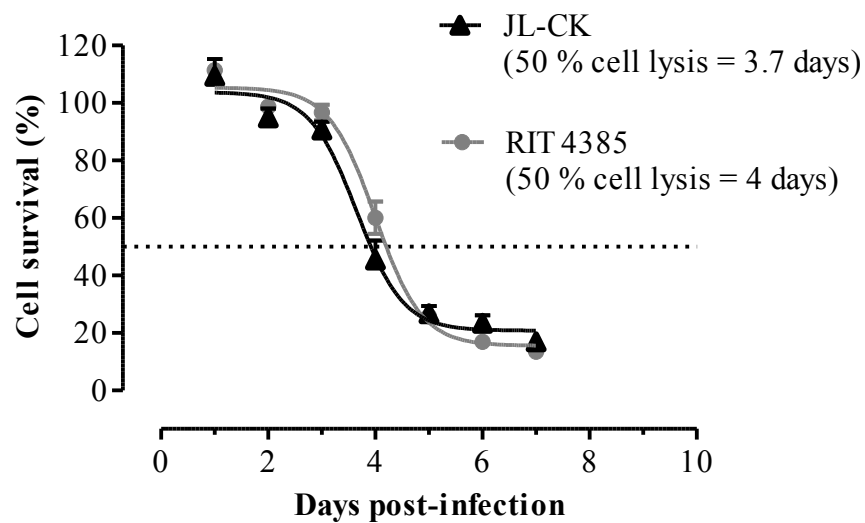


Figure 8: Vero cell lysis after infection with JL-CK or RIT 4385 viruses.

Cells were infected at a MOI of 0.0001 pfu per cell and cell viability determined daily by measuring the amount of MTS formazan produced by viable cells at 492 nm. Each timepoint was measured in triplicate. The error bars represent the standard error of the mean for each timepoint and the dotted line marks 50 % cell survival.

The 7 day timepoint progeny viruses were titrated in Vero cells to compare titres of both JL-CK and RIT 4385 with the results expressed as pfu per cell. The data in Figure 9 show that whilst both viruses share a similar cell lysis profile, by day 7 JL-CK produced at least 100 times more progeny virus. Analysis of the data by student's *t*-test identified the difference in progeny virus titre between the two groups to be significantly different where $p < 0.0002$, indicating efficient release of the JL-CK virus.

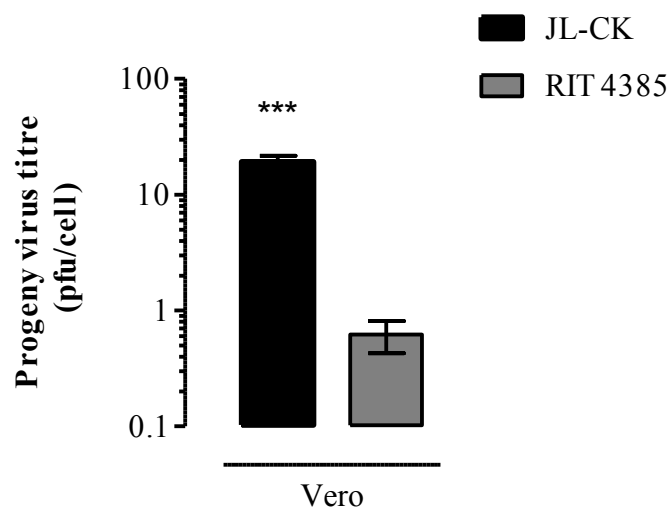


Figure 9: Progeny virus titres of JL-CK and RIT 4385 after 7 days incubation in Vero cells.

Cells were inoculated at a MOI of 0.0001 pfu per cell, incubated for 7 days before titration in Vero cells, in triplicate. The error bars represent the standard error of the mean for each group.

A student's *t*-test was applied to the data with a p value < 0.05 considered significant, $*** p < 0.0002$.

3.3.2 *Growth of JL-CK and RIT 4385 in MDCK cells*

The propagation of JL-CK MuV in primary canine kidney cells may have provided the virus with a preference for growth in canine kidney cells. In order to determine if there was a difference in virus growth and yield, JL-CK and RIT 4385 were inoculated at a MOI of 0.0001 pfu per cell on both Vero (simian kidney) and MDCK (canine kidney) cells and incubated for 3 or 7 days at 35 °C, 5% CO₂ (primary canine kidney cells were not available). The progeny viruses were titrated in Vero cells and the resulting plaques counted. Figure 10 shows the titres of progeny virus after 3 days growth in Vero and MDCK cells and Figure 11 shows the titres of progeny virus after 7 days growth.

JL-CK produced at least 10 times more progeny virus than RIT 4385 in both Vero (* $p = 0.0591$) and MDCK cells ($p = 0.1550$) after 3 days growth (Figure 10) and at least 100 times more progeny virus in both Vero (*** $p = 0.0002$) and MDCK cells (**** $p < 0.0001$) after 7 days growth (Figure 11). There was significant difference in JL-CK progeny virus titres between Vero and MDCK cells (**** $p < 0.0001$) suggesting that passage of JL-CK in primary canine kidney cells led to adaptation of the virus to this cell type. The difference in progeny virus titre between Vero and MDCK cells was also significant (* $p = 0.0100$) for the RIT 4385 virus, with a higher titre observed in Vero cells.

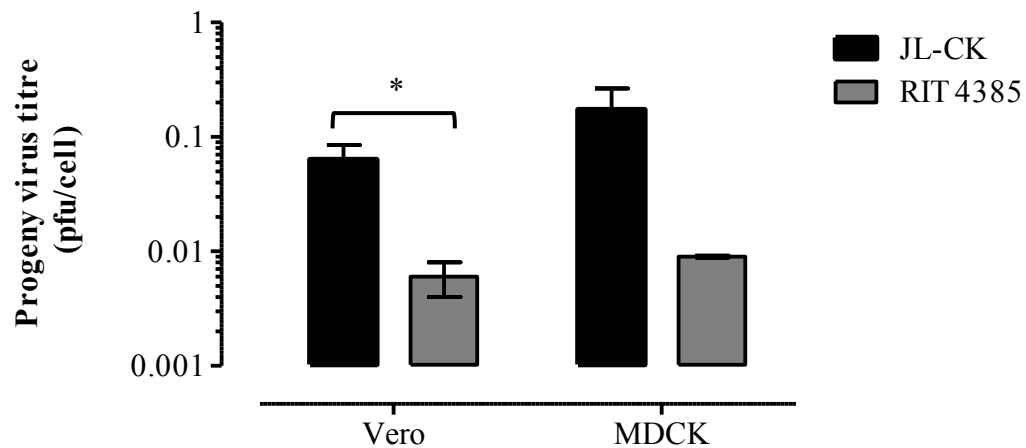


Figure 10: Progeny virus titres of JL-CK and RIT 4385 after 3 days incubation in Vero and MDCK cells.

Cells were inoculated at a MOI of 0.0001 pfu per cell and incubated for 3 days before titration in Vero cells, in triplicate. The error bars represent the standard error of the mean for each group. A student's *t*-test was applied to compare the data for the two viruses in each group, with a *p* value < 0.05 considered significant, Vero ^{*}*p* = 0.0591, MDCK *p* = 0.1550. Note, the Y-axis is represented in a log scale for visualisation, however the data is not log transformed.

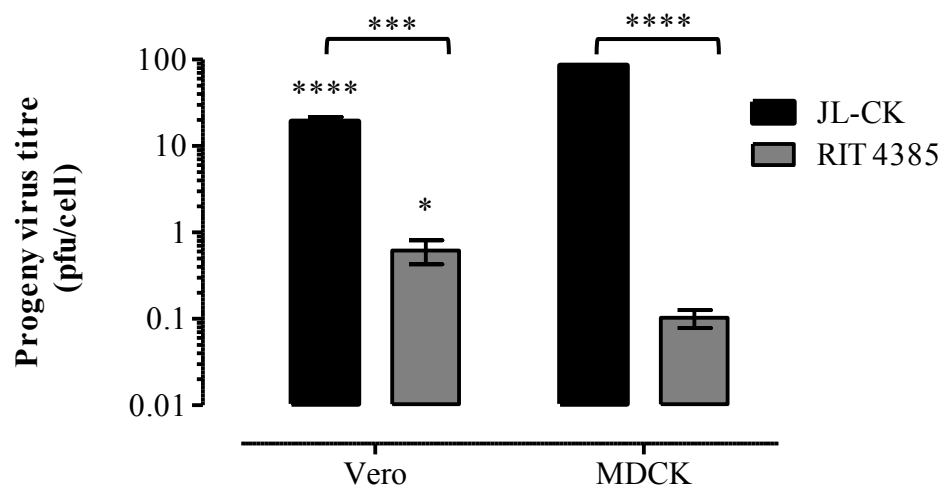


Figure 11: Progeny virus titres of JL-CK and RIT 4385 after 7 days incubation in Vero and MDCK cells.

Cells were inoculated at a MOI of 0.0001 pfu per cell and incubated for 7 days before titration in Vero cells, in triplicate. The error bars represent the standard error of the mean for each group.

A student's *t*-test was applied to compare the data for the two viruses in each group with a *p* value < 0.05 considered significant, ****p* = 0.0002, *****p* < 0.0001 and to compare the titre of each virus in the two cell lines *****p* < 0.0001, **p* = 0.0100. Note, the Y-axis is represented in a log scale for visualisation, however the data is not log transformed.

3.3.3 Comparison of progeny virus titres in Vero, MDCK and SH-SY5Y cells and media

To identify whether there was a difference in the titres of progeny virus budding from different cell types or species an assay was set up using Vero, MDCK and the human neuronal cell line, SH-SY5Y, which has been shown to enable MuV replication (Ninomiya et al., 2009; Rubin et al., 1998). Cells were infected at a MOI of 0.0001 pfu per cell, with either JL-CK or RIT 4385 MuVs and incubated. Cell lysates and supernatant were collected and titrated separately for each timepoint, to compare progeny virus titres. Figure 12 shows the virus titres at each timepoint for each cell type and corresponding supernatant.

The JL-CK virus produced higher titres of progeny virus compared to RIT 4385, in all cell lines. However the titres in the supernatants were in each case higher than those that were cell-associated, which was also seen with RIT 4385. The result also shows that by day 7, JL-CK produced more progeny virus in MDCK cells and supernatant compared to SH-SY5Y, which had titres that remained similar to those at day 4.

These results suggest that there is no preference for cell species i.e. simian, canine, and human, however JL-CK may display a preference for cell type (kidney). This also confirms our hypothesis that JL-CK and RIT 4385 display different *in vitro* characteristics, despite their shared origin.

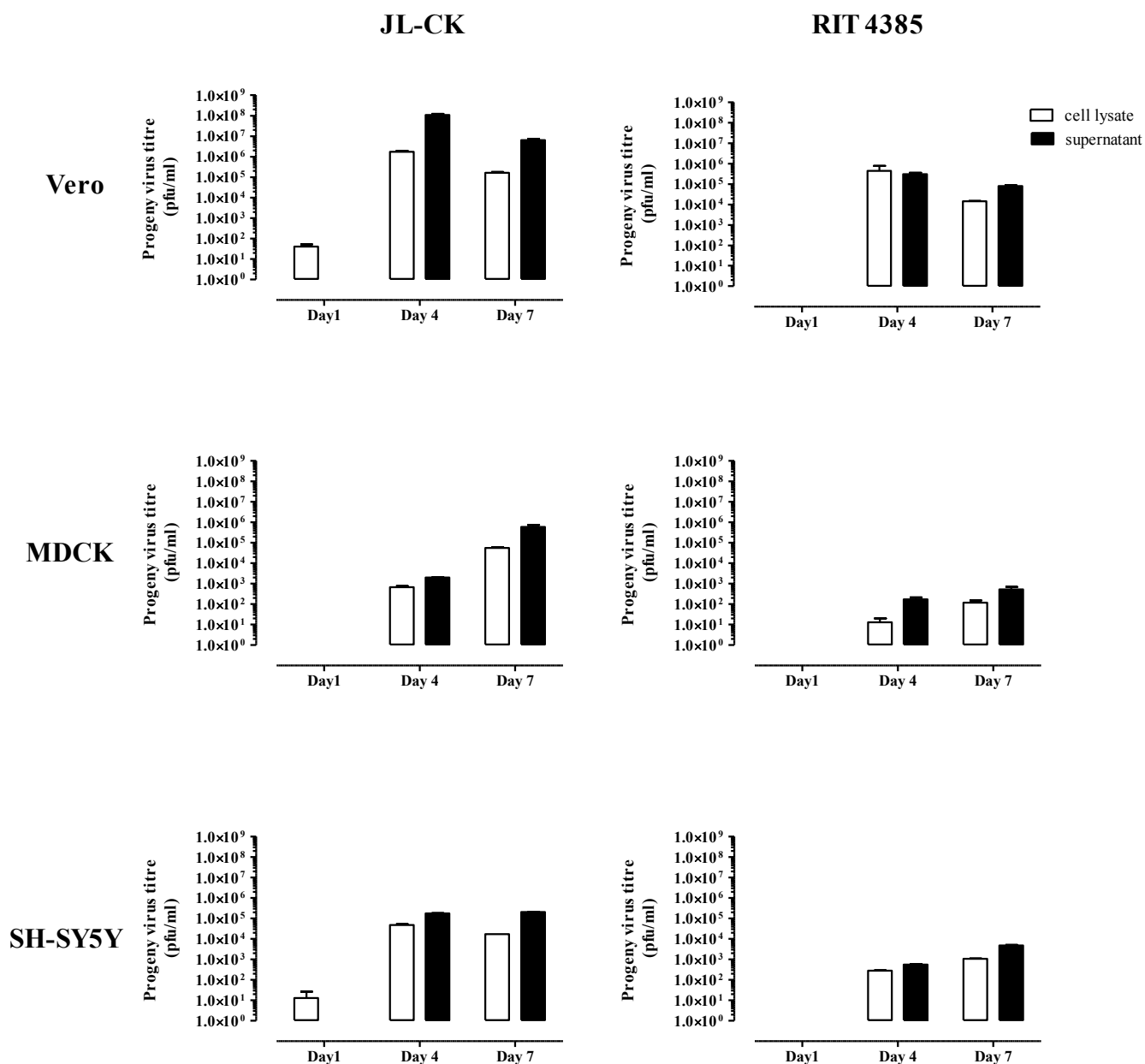


Figure 12: Comparison of JL-CK and RIT 4385 progeny virus titres from cell lysates and cell supernatants after growth in Vero, MDCK or SH-SY5Y cells.

Cells were inoculated at a MOI of 0.0001 pfu per cell, cell lysates and supernatants harvested at days 1, 4 and 7, followed by plaque assay in Vero cells. Left-hand panels represent JL-CK and right-hand panels represent RIT 4385. Cell lysates and supernatants from each timepoint were titrated in triplicate, the error bars represent the standard error of the mean.

3.4 *In vivo* characteristics

There currently is no animal model which mimics mumps disease. However there have been studies which showed that ferrets can generate a neutralising antibody response to MuV (Gordon et al., 1956; Parker et al., 2013). The antibody response against the JL-CK and RIT 4385 viruses was assessed in ferrets.

Two groups of male ferrets were pre-bled before being inoculated intra-nasally with either JL-CK (Ferrets A, B and C) or RIT 4385 (Ferrets D, E and F) MuVs. Over the course of the 28 day study, blood samples were collected at 7 day intervals before a terminal bleed at day 28. Ferrets were monitored throughout the study with temperature, weight, parotid gland(s) and testicle swelling measured. There were no clinical signs of disease in any of the ferrets. Serum from each timepoint was separated from red blood cells and used to detect neutralising antibodies.

3.4.1 *Plaque reduction neutralisation assays*

Plaque reduction neutralisation (PRN) assays measure the dilution of serum required to reduce plaque formation of a challenge virus by 50 % (ND₅₀) in cell culture. PRN titres are a function of the challenge virus strain used (Pipkin et al., 1999; Yates et al., 1996). Therefore, the serum from each ferret was challenged with the same virus that was inoculated into each ferret, i.e. sera from Ferrets A, B and C were challenged with the JL-CK virus and the sera from Ferrets D, E and F were challenged with the RIT 4385 virus. Each PRN assay had a positive control, which was the challenge virus without serum; the average plaque count for the positive control was 50 pfu. Figure 13 shows the neutralising antibody response expressed as ND₅₀ for each ferret over 28 days.

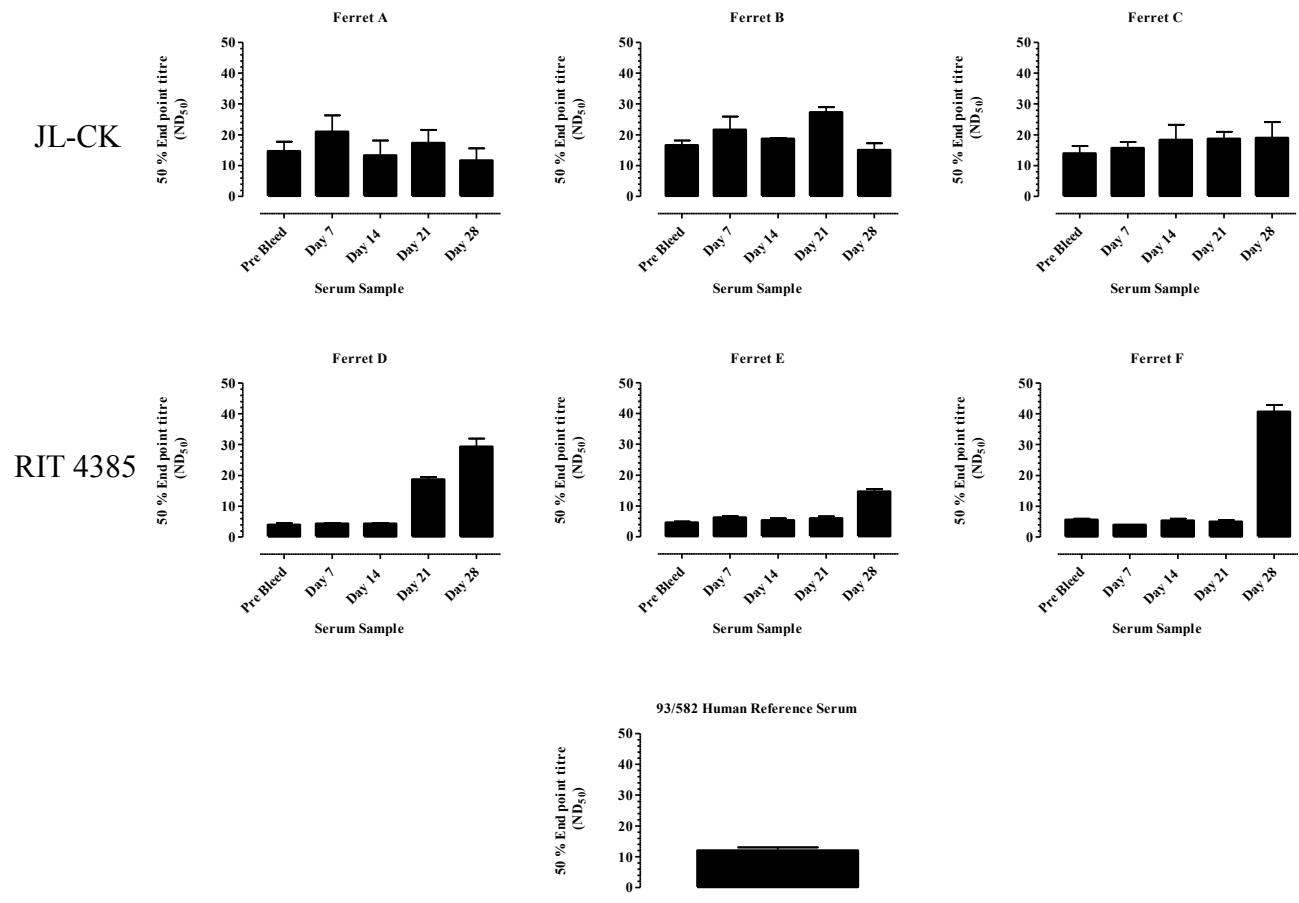


Figure 13: Neutralising antibody titres in ferret sera, produced in response to inoculation with JL-CK or RIT 4385 viruses.

Sera from each ferret was challenged with either the JL-CK (ferret sera A, B, C) or RIT 4385 viruses (ferret sera D, E, F), and the dilution required to reduce the number of plaques by 50 % (ND₅₀) was calculated. The human reference serum (93/582) was included as a control. All PRN assays were performed in triplicate and the error bars represent the standard error of the mean.

Ferrets A, B and C, which had been inoculated with the JL-CK virus, failed to neutralise the challenge JL-CK virus as the ND_{50} values at each timepoint were similar to those for the pre-bleed. Ferrets D, E and F which had been inoculated with the RIT 4385 virus, showed an increase in ND_{50} by day 28. The control human reference serum which was challenged with the RIT 4385 virus gave an average ND_{50} value of 12, which was lower than the day 28 ND_{50} values for the sera from the RIT 4385 inoculated ferrets.

MuV is serologically monotypic and immunisation with one strain is known to provide cross protection to other MuV strains. To identify if the neutralising antibodies produced by the ferrets could neutralise other MuVs, PRN assays were performed using different challenge MuVs and the day 28 sera, which contained the highest level of neutralising antibody. The serum from each ferret was challenged with the JL-CK, RIT 4385 or Mu90 MuVs. Figure 14 shows the ND_{50} values for the serum from each ferret after challenge.

Ferrets A, B and C that had been inoculated with JL-CK did not show any difference in ND_{50} when challenged with a related (RIT 4385) or representative wild-type (Mu90) MuV. The ND_{50} values after challenge with JL-CK produced similar results to those in Figure 13. Ferrets D, E and F, which had been inoculated with the RIT 4385 virus did show cross-protection, with higher ND_{50} values after challenge with the JL-CK virus, compared with the RIT 4385 virus. There was also a high neutralising response from Ferret F to all three challenge viruses, this ferret produced the highest ND_{50} value at day 28 (Figure 13), which may explain why this serum produced a better response to the Mu90 virus compared to ferrets D and E.

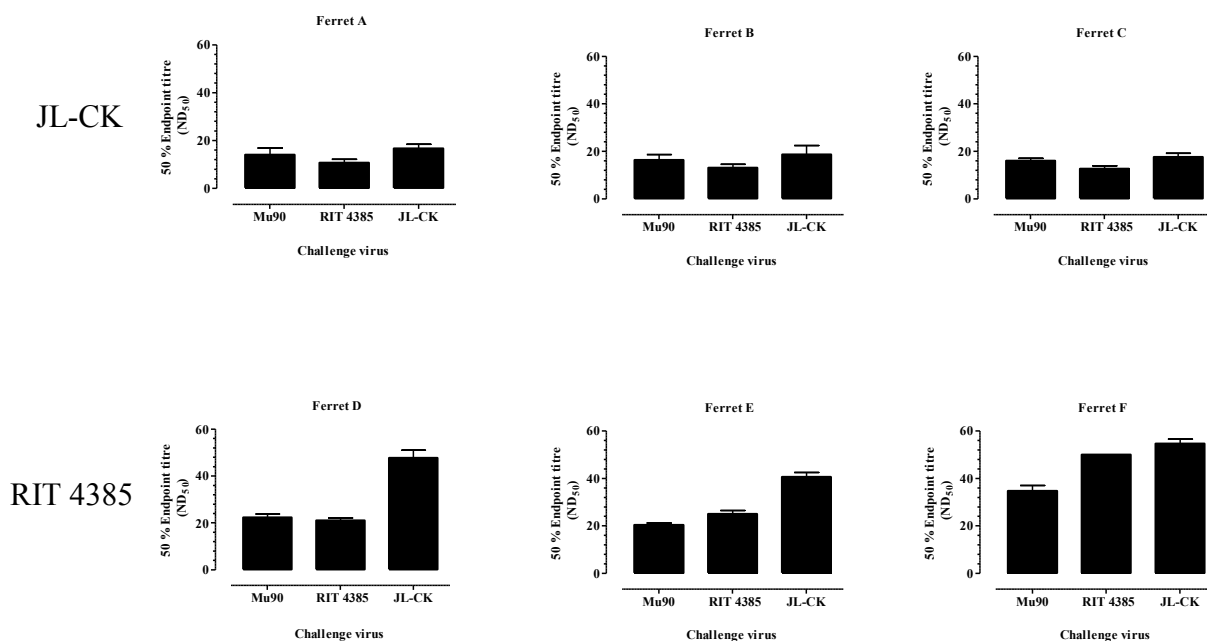


Figure 14: Neutralising antibody titres in day 28 ferret sera, produced in response to inoculation with JL-CK or RIT 4385 viruses.

Sera from each ferret was challenged with either the JL-CK, RIT 4385 or Mu90 viruses and the dilution required to reduce the number of plaques by 50 % (ND₅₀) was calculated. PRN assays were performed in triplicate and the error bars represent the standard error of the mean.

3.4.2 *Rat neurovirulence*

The neonatal rat model has been proposed for neurovirulence safety testing of MuV (Malik et al., 2009; Rubin et al., 2005). Groups of 22 neonatal rats were infected intra-cerebrally with JL-CK, RIT 4385 or Mu90 and monitored daily. At days 1, 4, 7 and 25 the brains from three animals in the JL-CK and RIT 4385 groups were removed for titration of virus. The remaining animals in each group were terminated at day 25. The brains were removed, fixed, sectioned and stained with haematoxylin and eosin (H&E). The cross-sectional areas of the whole brain (excluding cerebellum) and ventricles were measured using ImageJ (Schneider et al., 2012) and the results expressed as the percentage of the cross-sectional area of the whole brain (Rubin et al., 2005). Figure 15 shows a representative stained section from brains inoculated with either JL-CK or RIT 4385 MuVs.

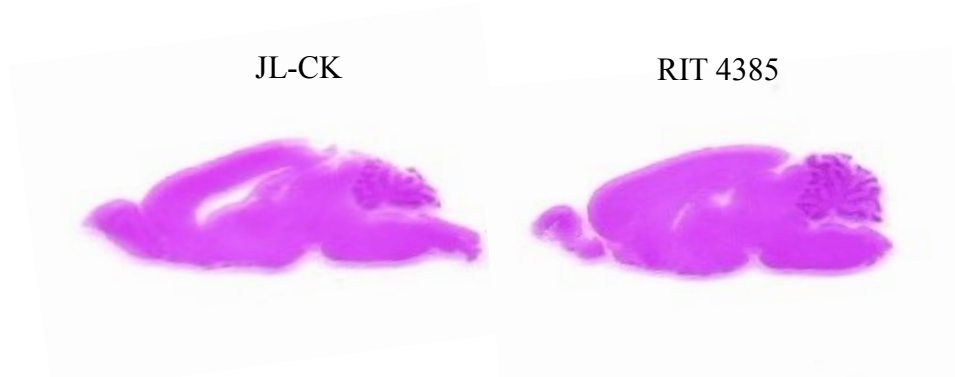


Figure 15: Representative sections from rat brains inoculated with JL-CK or RIT 4385 viruses.

Brains were fixed, cut through the anatomical midline, processed and embedded in paraffin wax with 10 μ m sections cut and stained with haematoxylin and eosin.

The brains from rats inoculated with the JL-CK virus showed larger ventricles compared to the brains from rats inoculated with the RIT 4385 virus. Figure 16 shows the results from individual rat brains. On average JL-CK displayed a higher neurovirulence potential compared to RIT 4385 ($p = 0.0035$ unpaired two-tailed t -test) and the negative control ($p < 0.0001$ unpaired two-tailed t -test), but when compared to Mu90, a neurovirulent, representative wild-type MuV (Rubin et al., 2005), the hydrocephalus observed in JL-CK was less ($p < 0.0001$ unpaired two-tailed t -test). We also observed significant variation between individual animals.

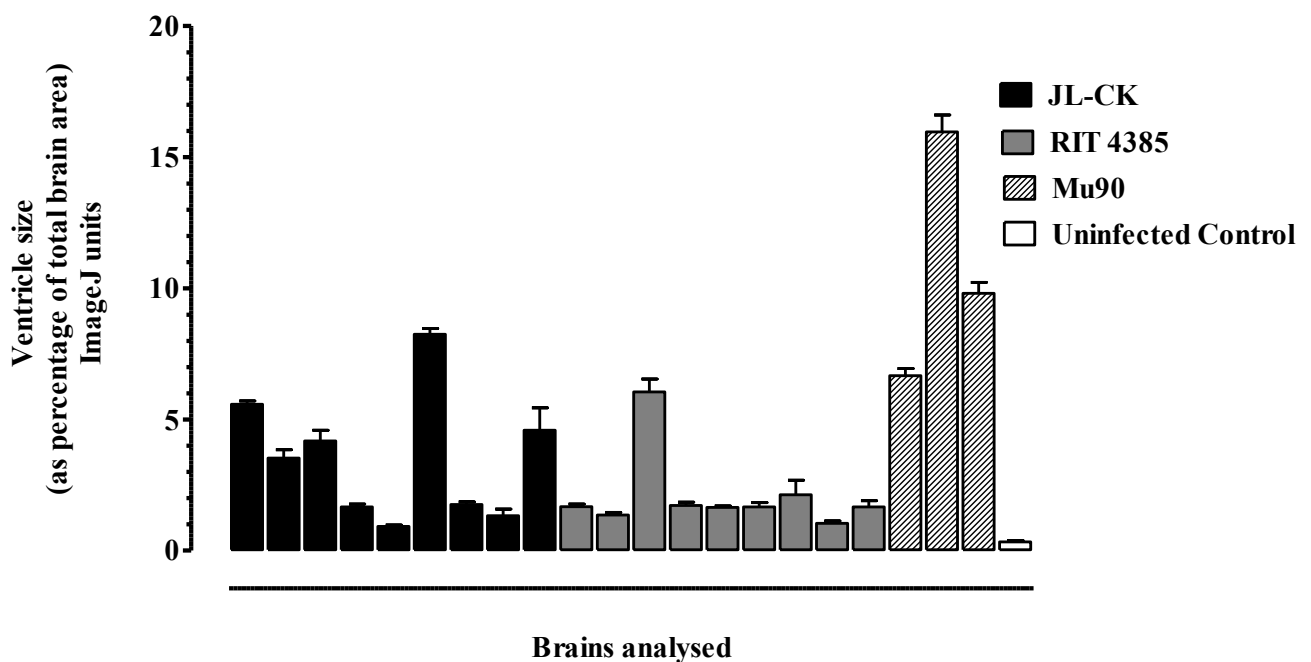


Figure 16: Ventricle size measured from individual rat brains inoculated with JL-CK, RIT 4385 or Mu90 viruses.

Each bar represents one rat brain, four sections were measured per brain and the error bars represent the standard error of the mean.

3.4.3 Virus titres in the rat brain

To compare progeny virus titres in the brains for the JL-CK and RIT 4385 groups, 20 % homogenates of the brains removed at days 1, 4, 7 and 25 were prepared. The brain homogenates were titrated in Vero cells. The titre of progeny virus expressed as pfu per ml of brain homogenate over time for each group is shown in Figure 17. The JL-CK virus yielded at least 10 fold more progeny virus by day 4 compared to RIT 4385. By day 7, the JL-CK yield dropped almost 10 fold, and the RIT 4385 progeny virus was undetectable. This phenomenon of progeny virus in rat brains peaking by day 4 has been observed previously (Sauder et al., 2011).



Figure 17: Progeny virus titres from rat brains inoculated with JL-CK or RIT 4385 viruses.

Brains were homogenised, clarified and serially diluted before titration in Vero cells, in triplicate. The error bars represent the standard error of the mean.

Both *in vitro* and *in vivo* data show that there are differences between the JL-CK and RIT 4385 viruses. To identify if these phenotypic differences are due to changes at the genomic level, both viruses were subjected to Sanger sequencing.

3.5 Genome analysis

3.5.1 Sanger sequencing of JL-CK and RIT 4385 vaccine viruses

Using the JL-5 MuV database sequence (accession AF201473) overlapping primers were designed to amplify both JL-CK and RIT 4385. Primers and their sequences are detailed in Table 9. RNA was extracted from both JL-CK and RIT 4385 and one-step RT-PCR was performed. PCR products were purified and sequenced before aligning against the JL-5 database sequence. The JL-CK virus genome contained 13 nucleotide positions, throughout the genome which showed variation to the database sequence. These translated into 10 predicted amino acid substitutions, which are depicted in Figure 18. The JL-CK amino acid sequences for each protein, highlighting the predicted substitutions aligned against the JL-5 sequences are detailed in Appendix 1.

Every gene contained at least one position which displayed a mixture of nucleotides and there was a mutation in the non-coding leader sequence. The N gene contained the most variation with four predicted amino acid substitutions plus one silent mutation. The Ter550Q amino acid substitution extends the nucleoprotein by 21 amino acids, which encompasses the L557P mutation. Interestingly the SH gene, which is known to be the most variable did not have any genetic variation to the database sequence. An example of the sequence chromatograms showing the mixture of nucleotides corresponding to the predicted amino acid substitutions E108D, Ter550Q and N56T is shown in Figure 19.

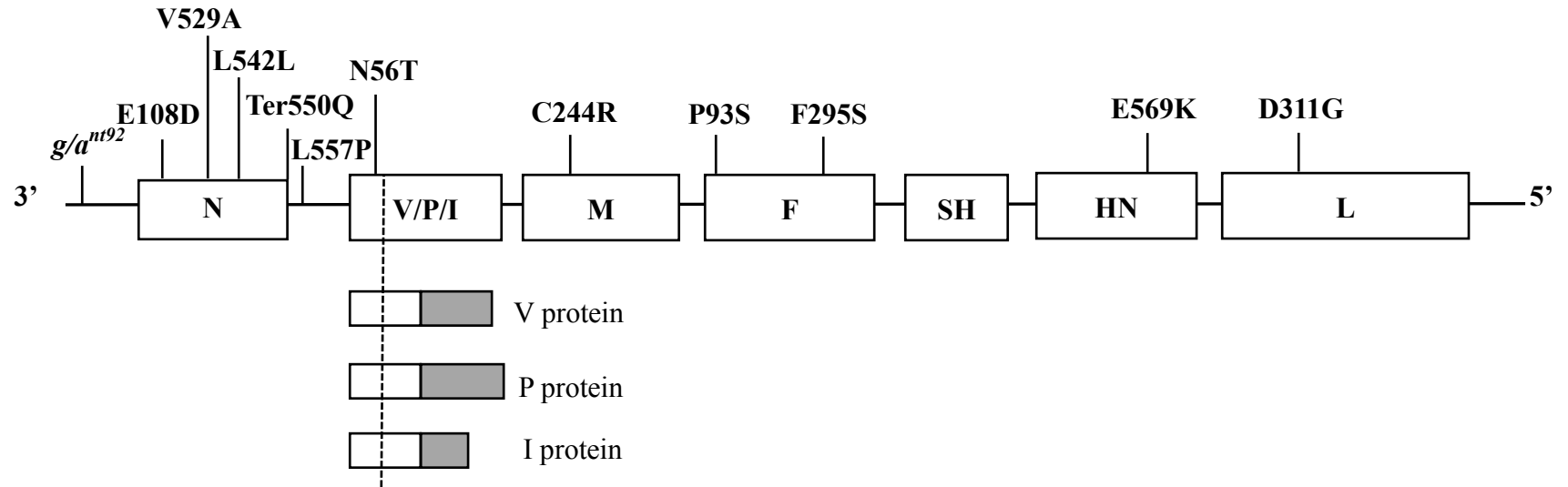


Figure 18: Schematic diagram of the JL-CK genome highlighting the predicted amino acid substitutions.

Consensus sequence from Sanger sequencing of JL-CK was compared and aligned to the JL-5 database sequence (accession AF201473) using ClustalW (EMBL-EBI).

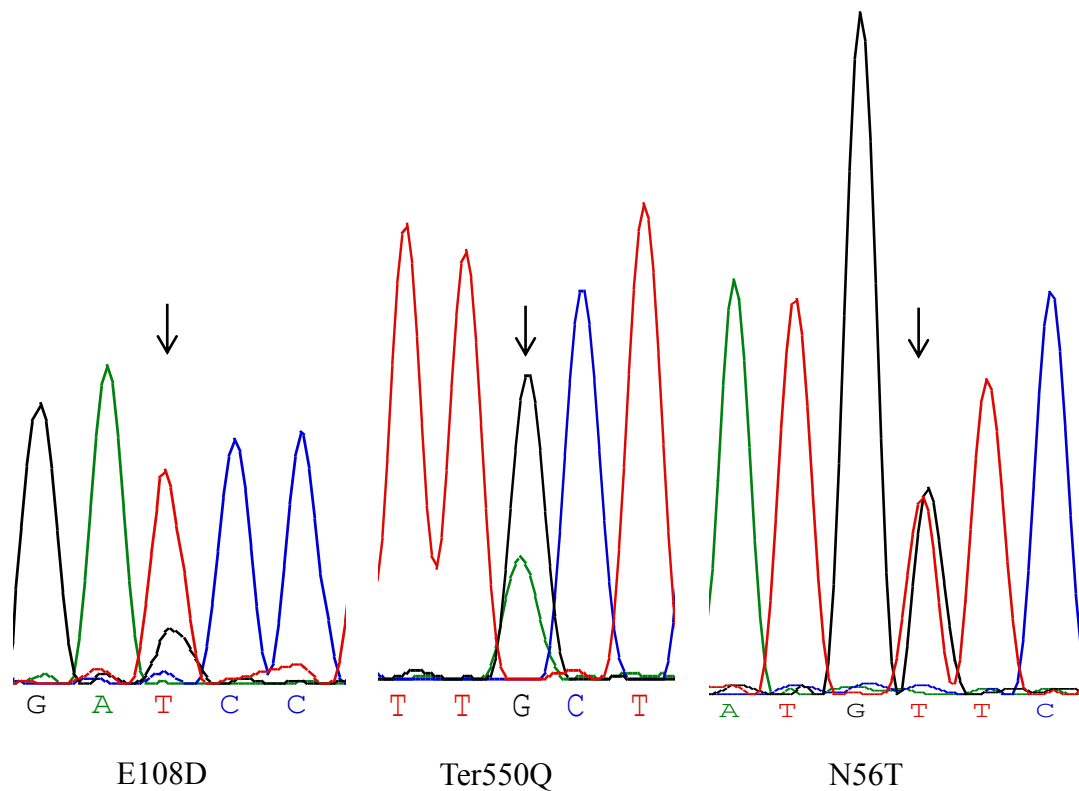


Figure 19: Example JL-CK sequence chromatograms identifying peaks which show a mixture of nucleotides.

The positions showing variation are marked with an arrow and correspond to the predicted amino acid substitutions E108D, Ter550Q and N56T.

The RIT 4385 genome also showed a small amount of variation compared to the JL-5 database sequence. There were two positions (nt1769 and nt1815) which displayed a mixture of nucleotides, these were previously unknown and correlated to one silent mutation in the N

protein (L542L), and one in the 5' non-coding region of the N gene, in this case. Both were also found in JL-CK (L542L and L557P).

The extent of variation in the JL-CK virus was unexpected, and is likely to be due to its propagation in primary canine kidney cells and the adaptation of the virus to the cell substrate.

In order to see if any of the mutations were specific to MuVs which have been propagated on primary canine kidney cells, a different MuV which had been produced in a similar manner was sequenced. The L3-SSW Pankow MuV is a Leningrad-3 strain which had been further passaged in primary canine kidney cells in the 1970's for use in the German Democratic Republic (Starke and Hlinak, 1974). However, it was withdrawn during clinical trials due to high incidence of aseptic meningitis (Tischer and Gerike, 2000). RNA extraction and RT-PCR using primers designed using the Leningrad-3 database sequence (accession AY508995), detailed in Table 10 produced PCR products covering the regions of interest. Sequence analysis did not detect any of the mutations observed in the JL-CK virus.

3.5.2 Comparison of JL-CK and RIT 4385 by MAPREC

To confirm the sequence data, the N and P genes of both JL-CK and RIT 4385 were subjected to Mutant Analysis by PCR and Restriction Enzyme Cleavage (MAPREC). The results of the MAPREC analysis are shown in Figure 20.

All mutations produced cleavage products in JL-CK, and the two mutations (L542L and L557P) identified in RIT 4385 were also confirmed by the presence of cleavage products. This confirmed that the sequence data was valid and not an artefact of the sequencing process.

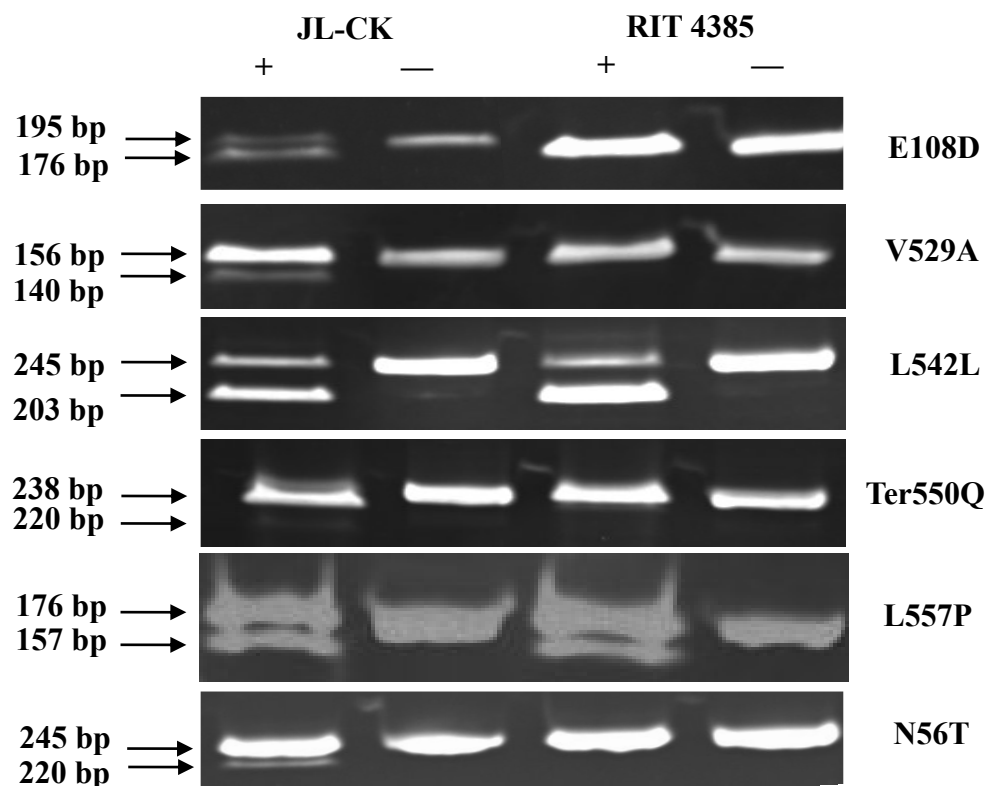


Figure 20: Analysis of the mutations in the N and P genes of JL-CK using MAPREC, compared to RIT 4385.

Digested (+) PCR products were run alongside undigested (-) PCR products, to allow for comparison and the identification of cleavage products. Mutations E108D, V529A, Ter550Q and N56T were confirmed in JL-CK (with L542L and L557P present in both viruses) by the identification of cleavage products after the addition of restriction enzyme.

3.5.3 *Nucleoprotein analysis by mass spectrometry*

The gene encoding the nucleoprotein shows the most variation between JL-CK and RIT 4385. One mutation in particular, Ter550Q, was of interest as it extended the C-terminus of the nucleoprotein by 21 amino acids. Purified JL-CK and RIT 4385 were subjected to polyacrylamide gel electrophoresis and Western blotting in order to identify the location of the nucleoprotein band(s) on a polyacrylamide gel and to confirm that the nucleoprotein is expressed. The protein profile of JL-CK and RIT 4385 on a 12 % polyacrylamide gel stained with colloidal blue is shown in Figure 21.

A replicate gel of Figure 21 was used to determine which bands corresponded to the nucleoprotein. This was determined by Western blotting using a polyclonal α -mumps nucleoprotein antibody raised in rabbits. Figure 22 shows the blot with chemiluminescent signal identifying the nucleoprotein at approximately 62 kDa.

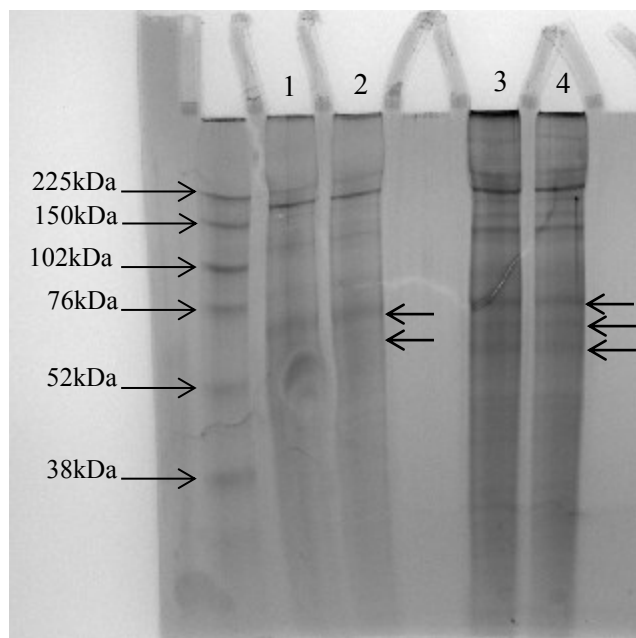


Figure 21: Polyacrylamide gel stained with colloidal blue showing the protein banding for JL-CK and RIT 4385.

Lanes 1 and 2 contain replicate samples of JL-CK virus concentrate and lanes 3 and 4 contain replicate samples of RIT 4385 virus concentrate, with the full-range Amersham Rainbow marker for size determination. The bands excised for mass spectrometry analysis are identified with arrows (two bands for JL-CK and three bands for RIT 4385), with both bands in each replica lane excised.

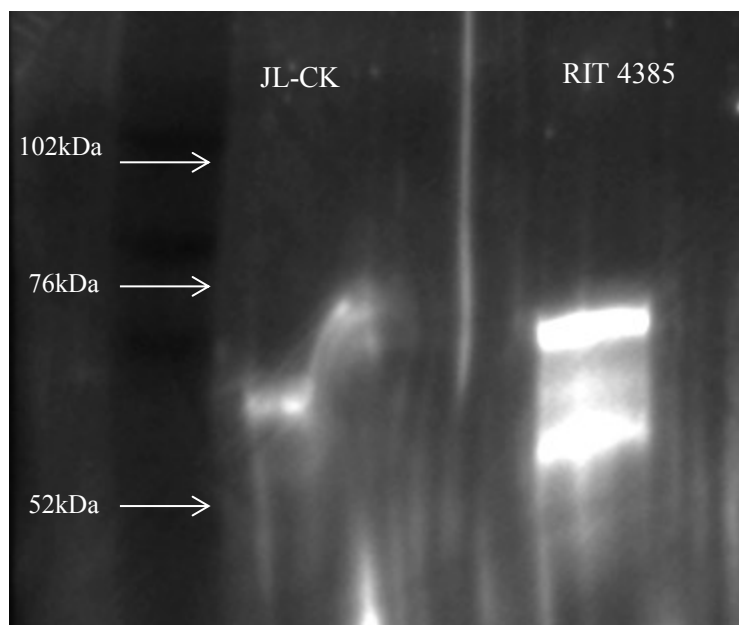


Figure 22: Detection of mumps nucleoprotein in JL-CK and RIT 4385 by Western blotting.

Primary antibody rabbit α -mumps nucleoprotein (1:10000) was incubated with the membrane followed by the secondary antibody goat α -rabbit HRP (1:2000). Mumps nucleoprotein was identified at approximately 62 kDa, using HRP chemiluminescence.

The purpose of the Western blot was to be as a guide to pinpoint the region of the colloidal blue-stained gel that contained the nucleoprotein, with an expected size of approximately 62 kDa. Unfortunately the blot did not transfer thoroughly as shown by the incomplete labelling pattern of the band(s) in the JL-CK lane. The multiple bands detected in the RIT 4385 sample were unexpected, most likely due to a combination of the polyclonal nature of the antibody and degradation of the nucleoprotein. The incomplete transfer, whilst unattractive, did not affect the downstream analysis which was performed on the colloidal blue-stained gel shown in Figure 21.

The preparation of samples and mass spectrometry was performed by Dr J. X. Wheeler, Laboratory for Molecular Structure, NIBSC. All visible bands between 52 kDa and 76 kDa (Figure 21) were excised, two from JL-CK and three from RIT 4385 before undergoing trypsin digestion. The tryptic peptides were extracted and subjected to online nano LC-MS/MS (Gaspari and Cuda, 2011) before identification by database searching.

The mass spectra produced were used to search the whole UniProt FASTA database in order to identify the peptides. All peptides identified were confirmed to be mumps nucleoprotein in both the JL-CK and RIT 4385 samples. The mass spectra produced from the JL-CK sample matched 9 peptides, with 190 residues identical to the 549 residues of the MuV (Jeryl Lynn) nucleoprotein (UniProt Q77158), corresponding to a sequence coverage of 34.6 %. The mass spectra produced from the RIT 4385 sample matched 10 peptides, with 214 residues out of 549 residues identical to the MuV (Jeryl Lynn) nucleoprotein (UniProt Q77158), this corresponded to a sequence coverage of 39 %. The additional peptide identified from the RIT 4385 sample was confirmed to be the nucleoprotein carboxy-terminal peptide (GEHVNTEPNPNQNAQLQVGDWDE). The sequence coverage map showing the matched peptides identified from the RIT 4385 sample and

compared to the UniProt database sequence (Q77158) of the MuV nucleoprotein is detailed in Appendix 2a. The mass spectra from the JL-CK sample did not identify the carboxy-terminal peptide. This suggested that the JL-CK nucleoprotein had a different carboxy-terminus to RIT 4385 and any other MuV nucleoprotein within the database. The sequence coverage map showing the matched peptides identified from the JL-CK sample compared to the UniProt database sequence (Q77158) of the MuV nucleoprotein is detailed in Appendix 2b.

The UniProt database was manually updated to include the putative additional JL-CK protein sequences (QITDMIKLTPIATIPGQSSHS) and (QITDMIKPTPIATIPGQSSHS), which had been predicted from Sanger sequence analysis. The mass spectra from both the JL-CK and RIT 4385 samples were searched against the updated database. This search identified 10 peptide matches from the JL-CK sample, corresponding to 203 residues out of 570 residues, (35.6 % sequence coverage). The carboxy-terminal peptide (LTPIATIPGQSSHS) of the putative extended nucleoprotein was identified in the JL-CK sample but not in the RIT 4385 sample.

The L557P mutation, which is located in the same peptide of the putative elongated nucleoprotein (PTPIATIPGQSSHS), was however not detected in the JL-CK sample. This is most likely due to the proline (P) disrupting the trypsin digestion motif therefore making the peptide too long to be sequenced. The identification of the carboxy-terminal peptide, LTPIATIPGQSSHS in the JL-CK sample but not in RIT 4385, confirmed that the elongated nucleoprotein, encoded by the Ter550Q mutation, is expressed at the protein level in the JL-CK MuV. The peptides identified from the RIT 4385 and JL-CK samples, mapped to the modified database entries for the MuV nucleoprotein are detailed in Appendices 2a and 2b.

CHAPTER 4 – IDENTIFICATION OF JL-CK VIRUS VARIANTS

The variation at the genomic level of the JL-CK vaccine virus was suggestive of multiple populations of MuV within the vaccine. Sanger sequencing of the unpassaged JL-CK virus identified 13 nucleotide polymorphisms, corresponding to four amino acid substitutions in the nucleoprotein; E108D, V529A, Ter550Q and L557P (Ter550Q extended the nucleoprotein by 21 amino acids, comprising the L557P mutation) plus one silent mutation (L542L). The shared region encoding proteins V/P/I contains one mutation, N56T. The matrix protein contains a C244R mutation, whilst the fusion protein contains two mutations P93S and F295S, which is based on two changes in adjacent nucleotides. The haemagglutinin-neuraminidase and large proteins both contain one mutation, E569K and D311G, respectively. Interestingly, no mutations were detected in the gene encoding the small hydrophobic (SH) protein, which is the most variable gene when comparing different mumps strains (Afzal et al., 1993) and used to classify MuV strains (Yeo et al., 1993). This indicated that if there is a mixture of MuVs within JL-CK, any virus isolated would be classified as the JL-5 strain and not any other MuV strain.

4.1 Isolation and sequence analysis of JL-CK variants

In order to identify whether there were more than one JL-5 virus present within the JL-CK vaccine, the JL-CK virus was plaque purified and subjected to Sanger sequencing. The resulting sequences from each plaque, covering the regions which displayed a mixture of nucleotides were aligned against the published sequence of JL-5. Analysis of 81 individual plaques identified five JL-CK variants plus the parent virus, which was identical to the published sequence of JL-5. The predicted amino acid changes, silent mutation and mutation in the non-coding leader sequence

that were identified in each variant, compared to the JL-5 sequence are depicted in Figure 23. The JL-CK variants contained a variety of the identified mutations, with JL-CK1 containing the least ($n = 3$) and JL-CK5 containing the most ($n = 9$). All of the JL-CK variants contained the non-coding leader mutation g/a^{nt92} plus the C244R and E569K mutations in the M and HN proteins. Each variant contained in addition at least one of the other mutations. JL-CK1 also contained a previously unidentified mutation, K36R in the N gene. Retrospective analysis of the plaque sequence data summarised in Table 14, revealed that 36 out of 79 plaques (45 %) contained this mutation, which had not been detected in the JL-CK vaccine vial by Sanger sequencing. The JL-CK2 variant contained the E108D mutation, identified in 6 out of 81 plaques (7 %), whilst JL-CK3 encompasses E108D plus Ter550Q, identified in 2 out of 81 plaques (2 %). The JL-CK4 variant contained E108D, Ter550Q, N56T, P93S and D311G, identified in 31 out of 81 plaques (38 %) whilst JL-CK5 included E108D, V529A, L542L, Ter550Q, L557P (in the extended nucleoprotein) and F295S identified in 3 out of 81 plaques (4 %). The virus with the identical sequence to JL-5 represented only 4 % of the population of plaques analysed. Some mutations appeared to be associated with each other, with g/a^{nt92}, C244R and E569K appearing together (* in Figure 23), as do N56T, P93S and D311G (# in Figure 23), and V529A, L542L, L557P and F295S (+ in Figure 23).

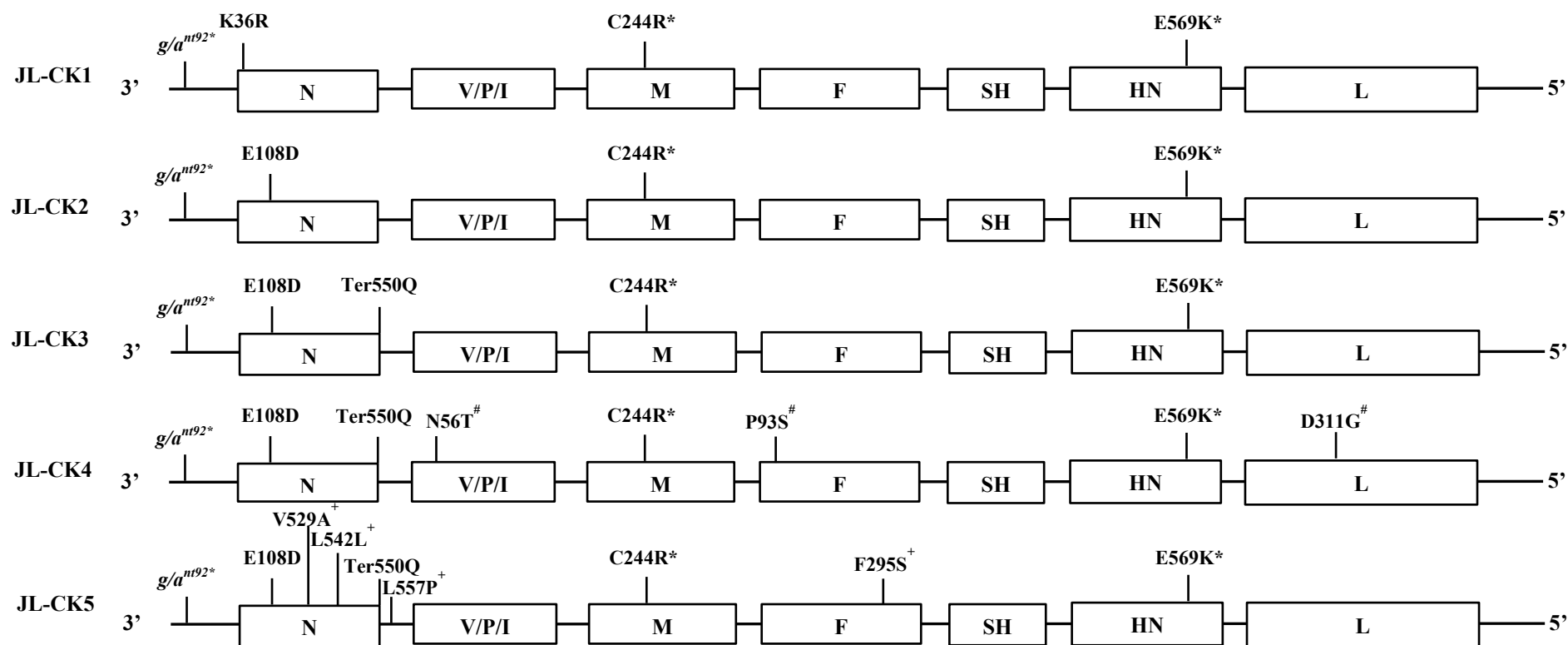


Figure 23: Schematic diagram of the JL-CK variants identified after plaque isolation and Sanger sequencing.

The specific mutations which appear to be associated with each other are depicted by * (g/a^{nt92} , C244R and E569K), [#] (N56T, P93S and D311G) or ⁺ (V529A, L542L, L557P and F295S)

Table 14: Mutations identified in each JL-CK variant and the frequency of each variant isolated from plaques.

Variant	Amino acid substitution							Frequency [#]
	Leader	N	V/P/I	M	F	HN	L	
JL-CK1	g/a ^{nt92}	K36R		C244R		E569K		45 %
JL-CK2	g/a ^{nt92}	E108D		C244R		E569K		7 %
JL-CK3	g/a ^{nt92}	E108D		C244R		E569K		2 %
		Ter550Q						
JL-CK4	g/a ^{nt92}	E108D	N56T	C244R	P93S	E569K	D311G	38 %
		Ter550Q						
JL-CK5	g/a ^{nt92}	E108D		C244R	F295S	E569K		4 %
		V529A						
		L542L						
		Ter550Q						
		L557P						
“Parental”	n/a	n/a	n/a	n/a	n/a	n/a	n/a	4 %

[#]frequency of each variant from 81 plaques sequenced, n/a – not applicable

4.2 Biological characteristics of each variant

To identify if a phenotype could be attributed to a particular JL-CK variant, each variant was assayed for its growth and plaque-forming ability *in vitro* and the ability to produce neutralising antibodies and neurovirulence potential *in vivo*.

4.2.1 *In vitro* growth of JL-CK variants

To identify if each JL-CK variant could grow on their own, without the presence of the other variants, and to compare the titres of the progeny viruses, Vero cells were infected with each

JL-CK variant and incubated. As in earlier experiments, Vero cells were inoculated at a MOI of 0.0001 pfu per cell and incubated. At each daily timepoint the progeny viruses were titrated in Vero cells and the results calculated as pfu per ml of cell lysate, Figure 24.

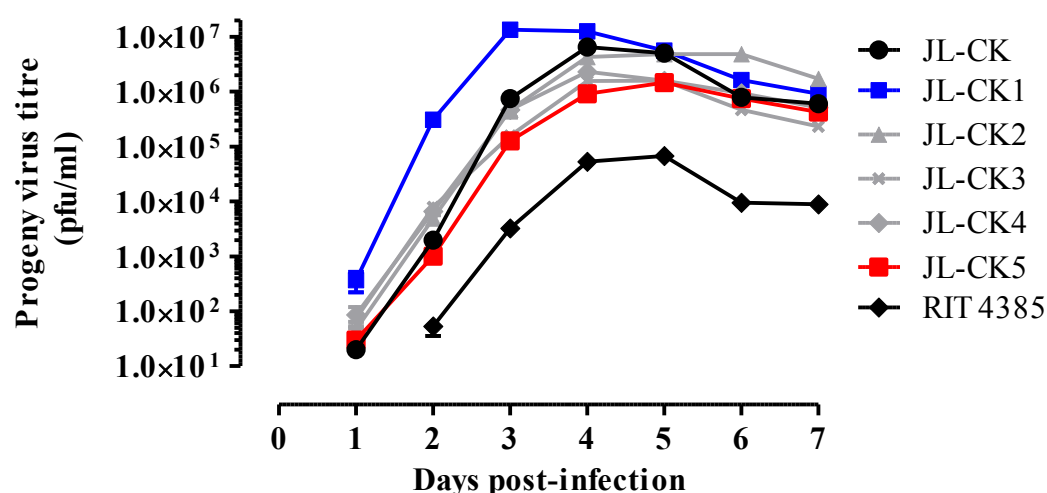


Figure 24: Progeny virus titres from individual variants incubated in Vero cells over 7 days.

Progeny viruses were titrated in Vero cells, in triplicate and plotted as pfu per ml of cell lysate. Error bars represent the standard error of the mean.

JL-CK1 produced the highest progeny virus titre (1.35×10^7 pfu per ml) in the shortest time (3 days post-infection). Compared to JL-CK and the other variants, JL-CK5 appeared to lag behind slightly at days 1 and 2 but did reach similar endpoint titres to JL-CK and the other variants by day 7, (JL-CK5 2.3×10^5 pfu per ml and JL-CK 6.1×10^5 pfu per ml). All JL-CK variants were capable of independent growth and each variant plus the JL-CK virus produced higher titres of progeny virus compared to RIT 4385.

4.2.2 *Plaque size comparison of JL-CK variants*

Each JL-CK variant was titrated in Vero cells to identify if the smaller plaque phenotype could be attributed to one particular variant or mutation. Representative plaque assays for each JL-CK variant plus the JL-CK and RIT 4385 viruses are shown in Figure 25.

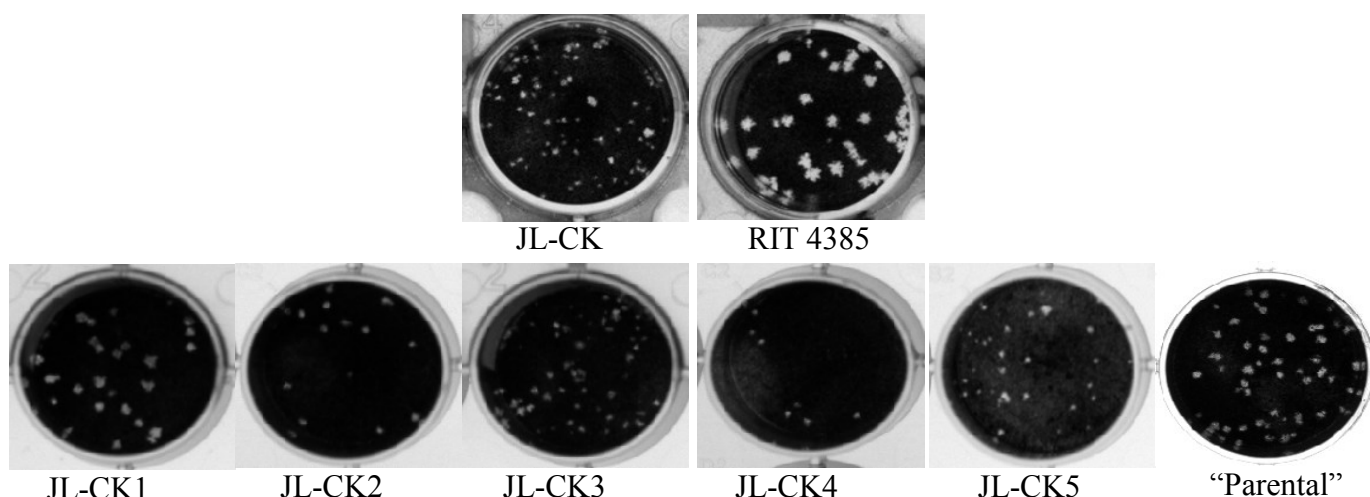


Figure 25: Representative images of plaques produced in Vero cells by JL-CK, RIT 4385 and each JL-CK variant.

The “Parental” panel is the parent-like JL-5 virus isolated from the JL-CK vaccine which did not contain any of the mutations identified in JL-CK.

The plaques produced by JL-CK3, JL-CK4 and JL-CK5 variants all appeared to display the smaller plaque phenotype. In order to quantify the plaque sizes, plaques from each variant plus the JL-CK and RIT 4385 viruses, were measured using ImageJ (Schneider et al., 2012). The results are shown in Figure 26. The data plotted is the number of plaques with corresponding plaque size, measured in ImageJ units.

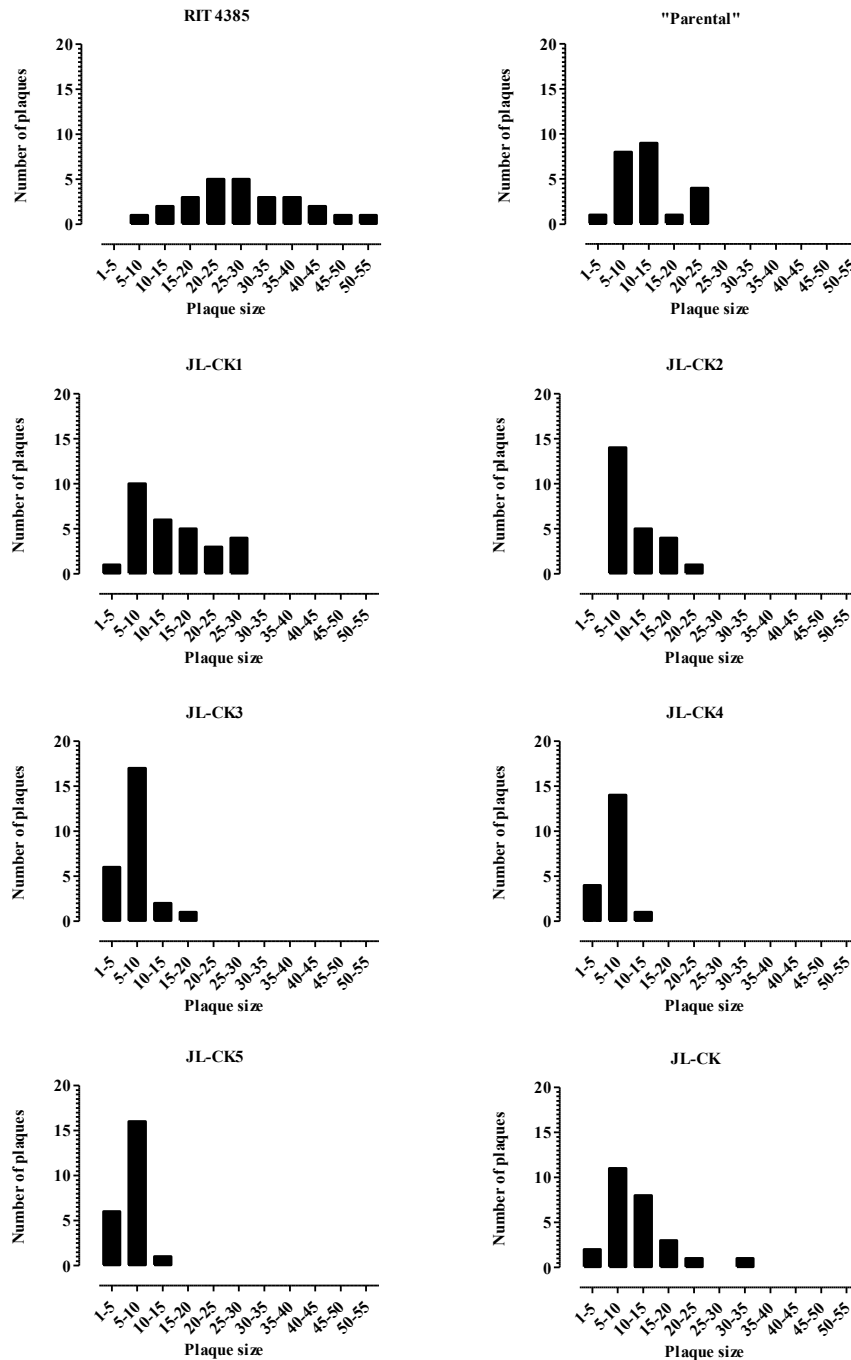


Figure 26: Comparison of plaque sizes produced by JL-CK, RIT 4385 and each JL-CK variant.

Plaque size measured using ImageJ, in terms of ImageJ units. The “Parental” panel is the parent-like virus isolated from the JL-CK vaccine which did not contain any of the mutations identified in JL-CK.

The smaller plaque phenotype was observed in the JL-CK3, JL-CK4 and JL-CK5 variants. The mutation that is unique to these variants is the Ter550Q mutation, which suggests that this mutation may play a role in this phenotype.

4.3 *In vivo* characteristics

Seven groups of male ferrets were pre-bled before being inoculated intra-nasally with either RIT 4385 (Ferrets 1, 2, 3), “Parental” virus (Ferrets 4, 5, 6), JL-CK1 (Ferrets 7, 8, 9), JL-CK2 (Ferrets 10, 11, 12), JL-CK3 (Ferrets 13, 14, 15), JL-CK4 (Ferrets 16, 17, 18) or JL-CK5 (Ferrets 19, 20, 21) MuVs. Over the course of the 28 day study, blood samples were collected at 7 day intervals before a terminal bleed at day 28. Ferrets were monitored throughout the study with temperature, weight, parotid gland(s) and testicle swelling measured. There were no clinical signs of disease in any of the ferrets. Serum from each timepoint was separated from the red blood cells, for use in the detection of neutralising antibodies by PRN assay.

4.3.1 *Plaque reduction neutralisation assays*

The serum from each ferret was challenged with the RIT 4385 virus. Each PRN assay had a positive control which was the challenge virus without serum added. The average plaque count for the positive control was 50 pfu. Figure 27 shows the neutralising antibody response expressed as ND₅₀ for the ferrets inoculated with either the RIT 4385 virus or the “Parental” virus over 28 days.

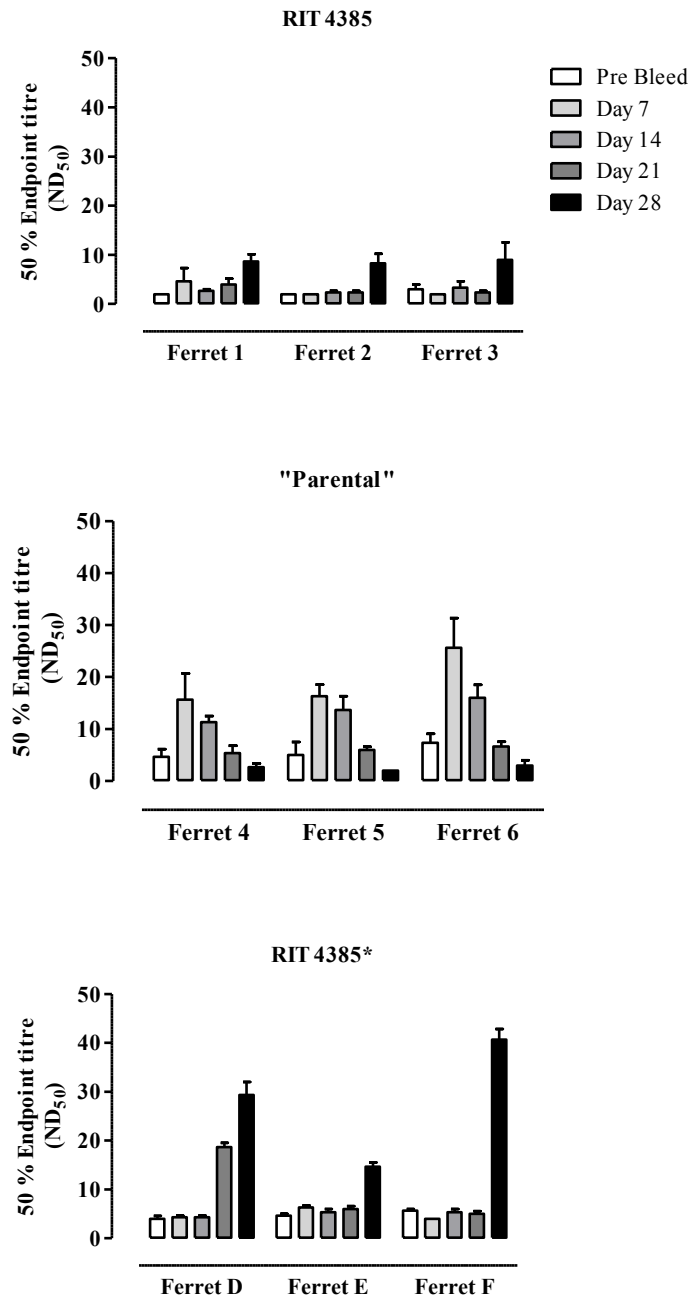


Figure 27: Neutralising antibody titres in ferret sera, produced in response to inoculation with RIT 4385 or the “Parental” virus isolated from the JL-CK vaccine.

Sera from each ferret were challenged with the RIT 4385 MuV and the dilution required to reduce the number of plaques by 50 % (ND₅₀) was calculated. PRN assays were performed in triplicate and the error bars represent the standard error of the mean. For comparison, RIT 4385* shows the ND₅₀ values from the RIT 4385 inoculated ferrets described in Figure 13.

The ND₅₀ values for Ferrets 1, 2 and 3 inoculated with RIT 4385 were low compared to the earlier PRN assays (Figure 13). This is most likely due to an error in the inocula which was found to be lower than that used in the earlier *in vivo* study. Therefore, for comparison the results from the previous PRN assay (RIT 4385*) described in Chapter 3 are included in Figure 27. Nevertheless a similar trend was observed; ferrets 1, 2 and 3 again showed an increase in ND₅₀ by day 28. In contrast, the “Parental” virus (Ferrets 4, 5 and 6) did not behave like RIT 4385. The ND₅₀ values reached their maximum titre at day 7 which reduced at day 14 to the levels of the pre-bleed samples by days 21 and 28. This could be a response to cellular debris left over from the “Parental” virus isolation and growth in Vero cells, rather than the virus. Despite variability between different animals, the results were comparable within each experimental group.

The ND₅₀ values from the sera obtained from ferrets inoculated with the remaining JL-CK variants are shown in Figure 28.

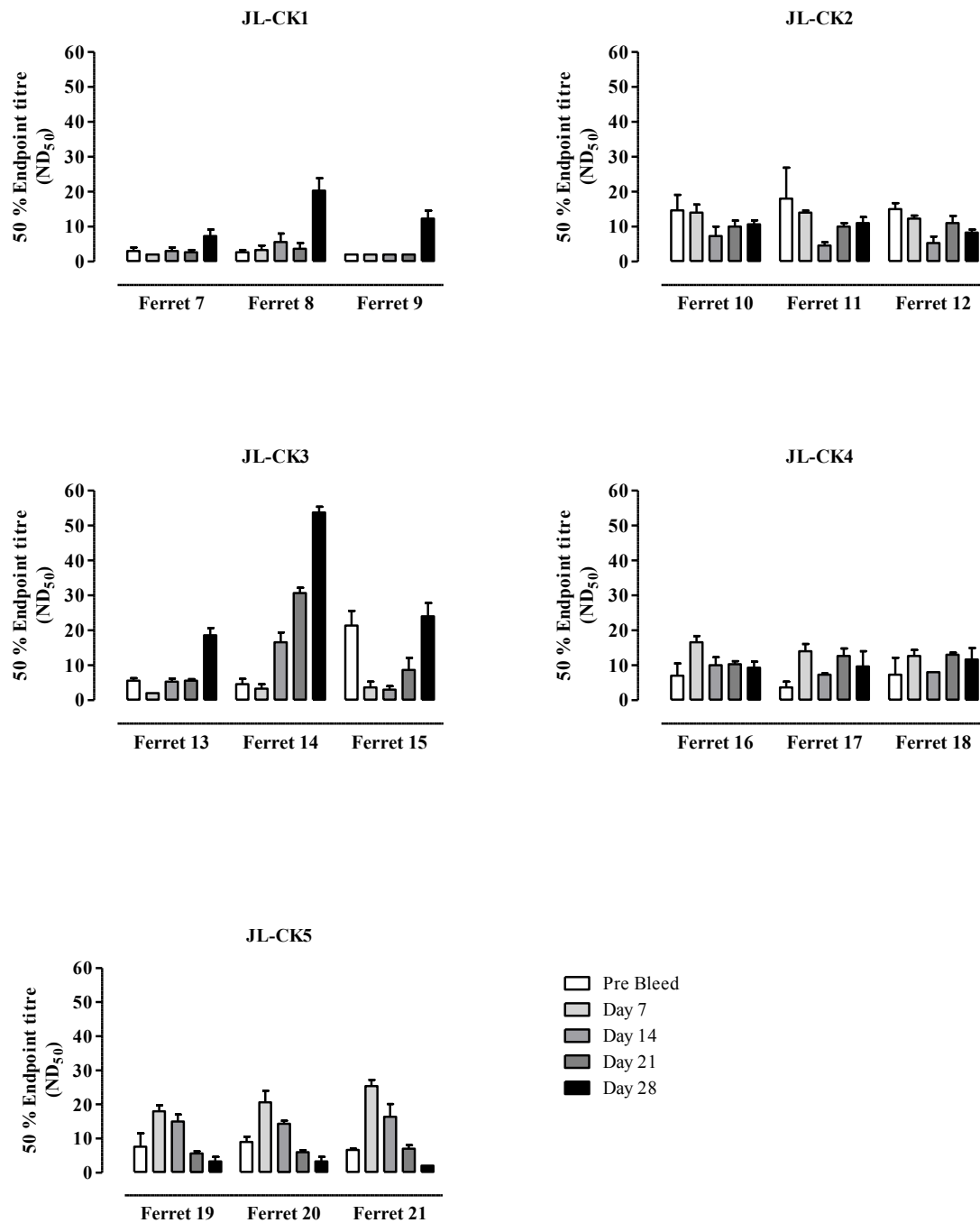


Figure 28: Neutralising antibody titres in ferret sera, produced in response to inoculation with JL-CK1, JL-CK2, JL-CK3, JL-CK4 or JL-CK5 variants.

Sera from each ferret were challenged with the RIT 4385 MuV and the dilution required to reduce the number of plaques by 50 % (ND₅₀) calculated. PRN assays were performed in triplicate and the error bars represent the standard error of the mean.

The ND₅₀ values from ferrets 7, 8 and 9, inoculated with the JL-CK1 variant showed a similar trend to those of RIT 4385, increasing by day 28. Ferrets 13, 14 and 15, inoculated with the JL-CK3 variant also showed an increase in neutralising antibody. Ferret 14 in particular produced the highest ND₅₀ value (ND₅₀ = 54), whilst ferret 15 was inconsistent, with a high pre-bleed (ND₅₀ = 21) making the data not comparable. The sera from ferrets 19, 20 and 21 inoculated with JL-CK5, behaved similarly to the sera from the ferrets inoculated with the “Parental” virus i.e. the maximum ND₅₀ value was obtained by day 7 then reduced at day 14, with days 21 and 28 showing similar ND₅₀ values to the pre-bleed sample. The sera from JL-CK4 inoculated ferrets were more variable. All three ferrets showed a slight increase in ND₅₀ values, similar to the “Parental” virus and JL-CK5 variant with the highest ND₅₀ observed at day 7. The serum from ferret 17 remained at a similar level throughout the study with a slight drop observed at day 14, regaining a similar value to day 7 by day 21. The pre-bleed from ferret 16 produced a raised ND₅₀ value, which was similar to those observed at days 14, 21 and 28. Ferret 18 produced a ND₅₀ of similar value at each timepoint so could not be compared. The sera from ferrets inoculated with JL-CK2 did not produce neutralising antibodies within the 28 day study with the ND₅₀ values lower than the pre-bleed values for each ferret.

There is considerable variation between each JL-CK variant and also between each ferret in some cases, but overall the sera from ferrets inoculated with RIT 4385, JL-CK1 and JL-CK3 appeared to neutralise the RIT 4385 virus with an increase in neutralising antibody titre by day 28. The sera from ferrets inoculated with JL-CK5 and two of the ferrets inoculated with JL-CK4 neutralised the RIT 4385 virus in a similar manner to the “Parental” virus (isolated from the JL-CK vaccine). This again could be a response to cellular debris in the inoculum, left over from the isolation and growth in Vero cells of the variants. There was no obvious association between individual mutations and ND₅₀ value.

4.3.2 Rat neurovirulence

Groups of six neonatal rats were infected intra-cerebrally with JL-CK, RIT 4385, JL-CK1, JL-CK2, JL-CK3, JL-CK4, JL-CK5 or the “Parental” virus isolated from the JL-CK vaccine, and monitored daily. At day 25, the rats were terminated and the brains were removed, fixed, sectioned and stained with H&E. The cross-sectional areas of the whole brain (excluding cerebellum) and ventricles were measured using ImageJ (Schneider et al., 2012) and the results were expressed as the percentage of the cross-sectional area of the whole brain. Figure 29 shows the results from individual rat brains.

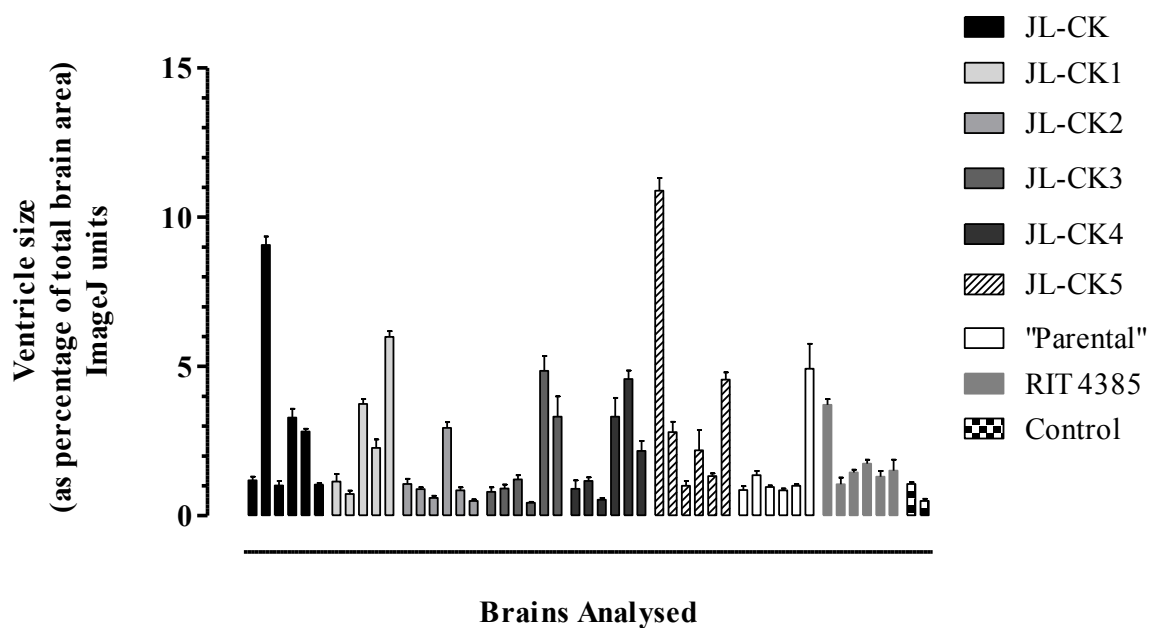


Figure 29: Ventricle sizes from individual rats inoculated with JL-CK, RIT 4385 or each individual JL-CK variant.

Each bar represents one rat with four brain sections measured for each. The error bars represent the standard error of the mean. Uninfected rat brains were used as a control.

There was considerable variation between animals in each group. Each bar represents the average measurement from four sections from one rat brain. At least 50 % of rats in the groups inoculated with JL-CK1, JL-CK4 and JL-CK5 variants showed an increase in ventricle size, suggesting an increase in neurovirulence potential, which was similar to the JL-CK inoculated rats. The ventricle sizes of the brains from rats inoculated with the “Parental” virus isolated from JL-CK, appeared similar to the RIT 4385 inoculated rats.

In order to compare each group, the ventricle sizes were plotted as one bar per group as shown in Figure 30.

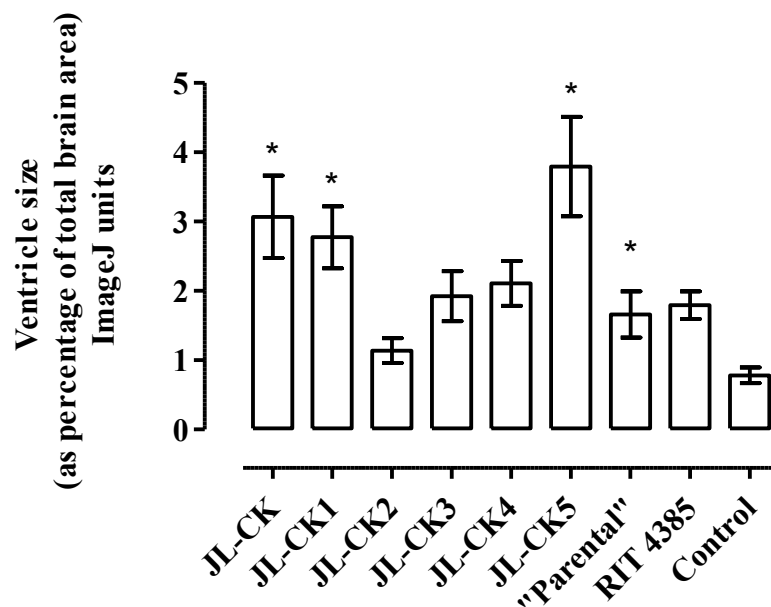


Figure 30: Ventricle sizes measured from rat groups inoculated with JL-CK, RIT 4385 or each individual JL-CK variant.

Each bar represents each group containing six rats. Four brain sections were measured for each rat and the error bars represent the standard error of the mean.

Each bar represents the average measurement from four sections from each rat brain in a group. The results for JL-CK and RIT 4385 were comparable to the data shown for a previous experiment (Figure 16). JL-CK1 and JL-CK5 showed a similar increase in ventricle size as the JL-CK virus, suggesting an increase in neurovirulence potential. When compared to RIT 4385, the p values obtained were; JL-CK, $p = 0.0478$ (*), JL-CK1, $p = 0.0406$ (*) and JL-CK5, $p = 0.0100$ (*). Compared to JL-CK, the “Parental” virus did not show an increase in neurovirulence potential, behaving like RIT 4385, as did JL-CK3 and JL-CK4, with p values of $p = 0.0440$ (*), $p = 0.1055$ and $p = 0.1627$ respectively. JL-CK2 showed significantly lower neurovirulence potential compared to both RIT 4385 ($p = 0.0181$) and JL-CK ($p = 0.0032$).

4.3.3 *Titres of progeny virus in rat brains*

In order to assess the growth of the JL-CK variants in the brain, groups of 16 neonatal rats were infected intra-cerebrally with JL-CK, RIT 4385, JL-CK1, JL-CK2, JL-CK3, JL-CK4, JL-CK5 or the “Parental” virus, isolated from the JL-CK vaccine and monitored daily. At days 1, 4, 7 and 10 the brains from three animals from each group were removed for titration of virus. The titre of progeny virus expressed as pfu per ml of brain homogenate over time for each brain is shown in Figure 31.

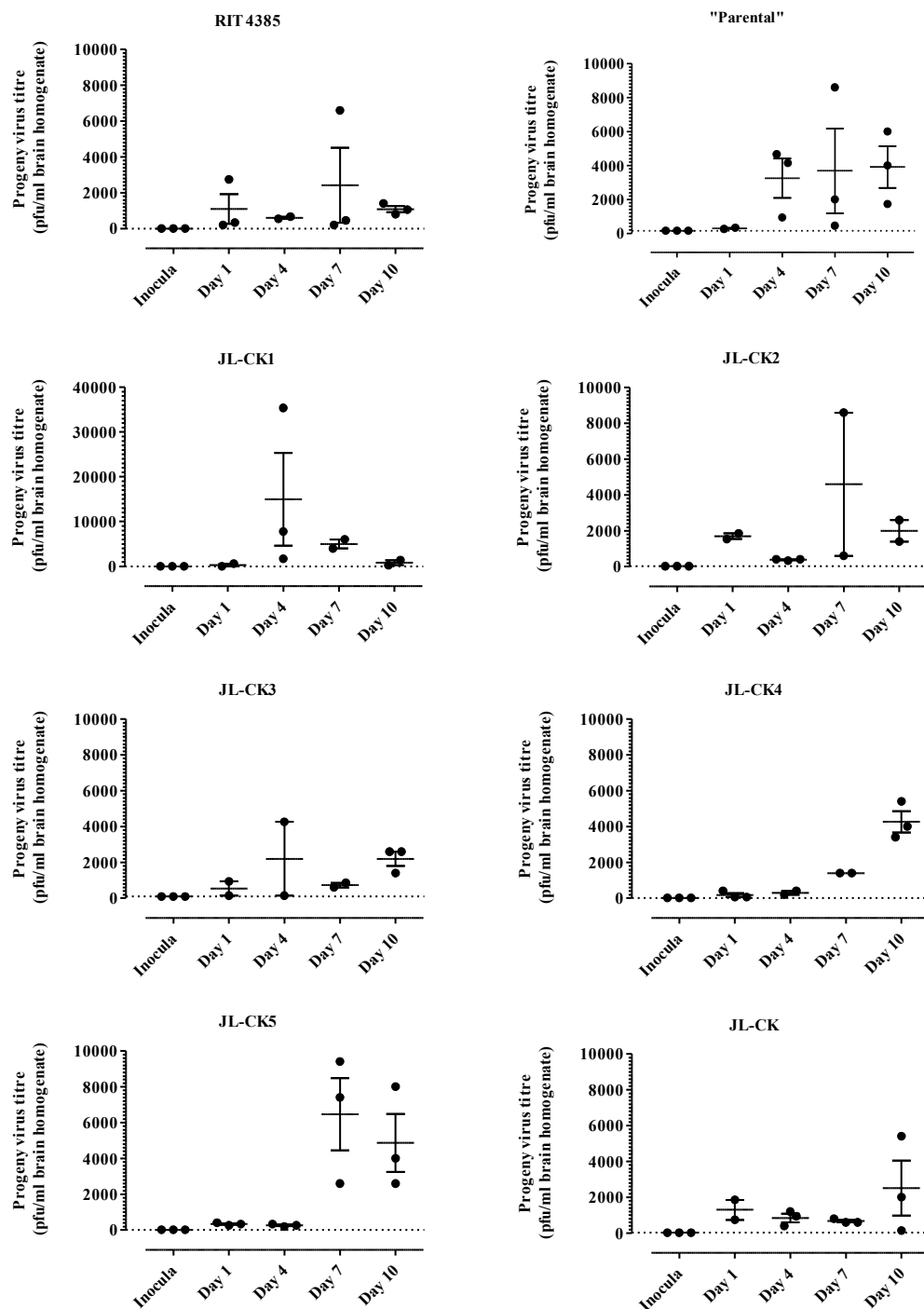


Figure 31: Progeny virus titres from rat brains inoculated with JL-CK, RIT 4385 or each individual JL-CK variant.

Each dot represents the progeny virus titre from one brain titrated in Vero cells, in triplicate.

The error bars represent the standard error of the mean. Note, data for the JL-CK1 variant graph are shown using a different y-axis compared to the other variants.

Each dot represents the average progeny virus titre from one brain performed in triplicate plaque assays, with three animals per timepoint. Again there was considerable variation between animals, shown by the range of the error bars. All JL-CK variants grew in the rat brain, reaching their maximum yield at different timepoints. JL-CK1 and JL-CK3 both reached their maximum progeny virus yield by day 4, producing average titres of 1.5×10^4 pfu per ml and 2.2×10^3 pfu per ml respectively, one rat in the JL-CK1 group produced the highest progeny virus titre of 3.5×10^4 pfu per ml at day 4. The RIT 4385, JL-CK2, JL-CK5 and the “Parental” viruses all reached their maximum yield by day 7, with average titres of 2.4×10^3 pfu per ml, 4.6×10^3 pfu per ml, 6.5×10^3 pfu per ml and 3.7×10^3 pfu per ml produced respectively. The maximum yield for the JL-CK4 variant and JL-CK virus progeny were not observed until day 10 with titres of 4.3×10^3 pfu per ml and 2.5×10^3 pfu per ml respectively. The yield of JL-CK and RIT 4385 progeny virus in this study is higher than in the previous *in vivo* study (Figure 17), although the maximum titre was reached at day 7 (RIT 4385) and day 10 (JL-CK) and the titres of each were more similar. Compared to the initial study, the average yield of JL-CK progeny virus at day 4 in this study was 847 pfu per ml compared with 1.4×10^3 pfu per ml in the earlier study and the average yield of RIT 4385 progeny virus at day 4 in this study was 600 pfu per ml compared with 31 pfu per ml at the same point in the earlier study.

CHAPTER 5 – COMPARISON OF JL-CK AND RIT 4385 BY DEEP SEQUENCING

MuV has been shown to adapt to various cell lines and it is this adaptation to cell lines which forms the basis of MuV attenuation for vaccine production. It has also been suggested that Vero cells may alter MuVs (Afzal et al., 2005; Parker, 2013). To identify whether the virus populations identified in plaques formed in Vero cells after a 10 day incubation period are represented at similar ratios in the unpassaged JL-CK vaccine, deep sequencing of JL-CK and RIT 4385 was performed.

5.1 Shotgun sequencing approach

Oligo dT cDNA produced from RNA extracted from unpassaged JL-CK was used as a template to amplify 5 kb fragments using primers pairs Pav73fw:Pav48rev, Pav47fw:Pav80rev, Pav55fw:Pav20rev and Pav25fw:Pav34rev as detailed in Table 9. However, none of the primer combinations produced fragments of the correct size despite adjusting the PCR parameters. To confirm that the template cDNA was suitable for amplification, primers Pav1fw and Pav2rev were used to successfully generate a 1 kb amplicon. Using the same cDNA, primers Pav73fw and Pav36rev were used to attempt the amplification of a 3 kb fragment, however the same PCR conditions as the Pav1fw:Pav2rev reaction (increasing the extension time from 1 min to 3 min) failed to produce a 3 kb amplicon. The fact that no amplification from the oligo dT cDNA was observed, was an obvious outcome in hindsight, as there are no poly A tails in the genomic RNA for the oligo dT to bind to in the cDNA synthesis step.

To increase the chance of producing MuV cDNA from the JL-CK RNA, a gene-specific primer approach was used to generate cDNA. The Pav34rev primer was used to produce

cDNA. However amplification using primers Pav73fw and Pav48rev and the PCR conditions as before, failed to produce a 5 kb amplicon.

Using shotgun sequencing for deep sequencing the JL-CK virus from the vaccine vial does not appear to be a feasible approach. It is possible that whilst RNA was extracted from the vaccine virus it may not contain a high enough concentration of good quality genetic material to allow amplification of 5 kb fragments even when the MuV genome is targeted.

5.2 Amplicon-based deep sequencing of unpassaged JL-CK and RIT 4385

Random hexamer cDNA was produced from RNA extracted from unpassaged JL-CK and RIT 4385 viruses and used as a template. Amplicons covering the regions containing the mutations, as shown in Figure 23 were generated using the fusion primers detailed in Table 13, and libraries prepared for sequencing as detailed in 2.5.2. The resulting sequences were aligned against the database sequence for JL-5 and the frequencies of each mutation established. The frequencies of each mutation determined by plaque isolation of JL-CK compared to the frequencies obtained by deep sequencing of unpassaged JL-CK and RIT 4385 are shown in Table 15.

The rank order of the majority of mutations in JL-CK remained consistent between the two methods. However, the data suggested that not all variants in the JL-CK vaccine behaved equally in Vero cell culture. The frequencies of the four mutations V529A, L542L, L557P and F295S that are unique to the JL-CK5 variant were all reduced ~ 6.8 fold in Vero cells, compared with unpassaged JL-CK virus. The E108D and Ter550Q mutations, which are present in variants JL-CK2, JL-CK3, JL-CK4 and JL-CK5, were also less prominent after growth in Vero cells, but only reduced ~ 2 fold. The K36R mutation in the nucleoprotein which was not identified during Sanger sequencing of the unpassaged JL-CK virus, was

detected at low frequency (9.1 %) by deep sequencing. This mutation whilst present at low levels in unpassaged JL-CK vaccine increased 5 fold after passage in Vero cells. The C244R and E569K mutations that are present in all JL-CK variants occurred as frequently in the unpassaged JL-CK vaccine and in the plaques produced in Vero cells, as did the non-coding leader g/a^{nt92} and the N56T, P93S and D311G mutations, which are unique to the JL-CK4 variant.

Table 15: Comparison of mutation frequencies detected in JL-CK plaques and by deep sequencing of unpassaged JL-CK and RIT 4385 viruses.

Nucleotide Position	Protein	Amino acid substitution	Mutation Frequency (%)		
			JL-CK Plaques [#]	JL-CK Unpassaged	RIT 4385 Unpassaged
92	-	Leader	98 ^a	96.70	n.d.
252	N	K36R	46 ^b	9.10	n.d.
469	N	E108D	51	86.60	n.d.
1731	N	V529A	4	27.00	6.20
1769	N	L542L	4	26.20	16.60
1793	N	Ter550Q	44	74.20	n.d.
1815	N	L557P	4	26.50	21.30 ^c
2145	V/P/I	N56T	38	40.50	n.d.
3993	M	C244R	96	97.50	n.d.
4822	F	P93S	38	45.30	n.d.
5429/30	F	F295S	4	27.3	n.d.
8318	HN	E569K	96	96.90	n.d.
9369	L	D311G	38	43.40	n.d.

[#]81 plaques were sequenced except ^a 49 plaques and ^b 79 plaques due to lack of material.

^c Mutation in non-coding region. n.d. not detected

The results demonstrated that the growth of JL-CK in Vero cells alters the virus population dynamics, preferentially selecting the JL-CK1 and JL-CK4 variants.

The results of deep sequencing the unpassaged RIT 4385 virus are also shown in Table 15. Three of the mutations identified in JL-CK are also present in RIT 4385; V529A at 6.2 % frequency, L542L at 16.6 %, and L557P at 21.3 %. The Ter550Q mutation was not detected in RIT 4385, indicating the L557P mutation is not expressed in this virus. This confirms the earlier Sanger sequencing and MAPREC data. These mutations have not previously been reported for this vaccine.

5.3 Deep sequencing of JL-CK and RIT 4385 after growth in Vero and MDCK cells

The difference in progeny virus titres described in Chapter 3 showed that the JL-CK virus produced a higher yield of progeny virus in both Vero and MDCK cells compared to RIT 4385. To investigate how the population dynamics change during growth in these cell lines, deep sequencing was performed on RNA extracted from cell lysates after Vero or MDCK cells were infected at a MOI of 0.0001 and incubated until CPE was observed (3 days post-infection). Table 16 shows the frequencies of mutations identified in unpassaged JL-CK, compared to those detected in cell lysates after 3 days incubation in Vero or MDCK cells or from plaques formed on Vero cells after 10 days incubation.

After 3 days incubation in Vero cells the frequencies for mutations V529A, L542L, L557P and F295S, which are unique to the JL-CK5 variant, decreased by 2.6, 2.8, 2.7 and 2.5 fold respectively. The frequency of the K36R mutation, which is unique to JL-CK1 increased 3 fold and the frequencies of mutations N56T, P93S and D311G which are unique to the JL-CK4 variant remained at similar levels. This suggests that Vero cells do not support the growth of the JL-CK5 variant. The JL-CK1 and JL-CK4 variants appeared to be preferentially selected in Vero cells over the other variants, even after 3 days post-infection.

This was in agreement with the results shown in Table 15 and demonstrated that by the time CPE was observed in Vero cells, there was already a change in the JL-CK virus population.

In MDCK cells on the other hand, all mutation frequencies remained consistent with those of the unpassaged JL-CK virus, the exception being the K36R mutation, which increased 2 fold. This confirmed that different cell substrates have different effects on MuV and suggested that MDCK cells support the growth of all JL-CK variants, in particular the JL-CK1 variant.

The frequencies of mutations identified in RIT 4385 after 3 days incubation in Vero or MDCK cells are shown in Table 17.

In the case of the RIT 4385 virus, the three mutations that are common to both vaccine viruses (V529A, L542L and L557P) were found at similar frequencies after incubation in Vero and MDCK cells. This suggested that the RIT 4385 virus is more stable in these cell lines than the JL-CK virus.

Table 16: Mutation frequencies identified by deep sequencing of JL-CK.

Mutation frequencies of unpassaged virus and Vero and MDCK cell lysates 3 days post-infection (in duplicate), in comparison to the frequency observed by Sanger sequencing of plaques formed in Vero cells 10 days post-infection.

Nucleotide position	Protein	Amino acid substitution	Mutation frequency (%)					
			Unpassaged	Vero 3 days p.i.		MDCK 3 days p.i.		Vero 10 days p.i.
92	-	Leader	96.70	94.7	94.3	72.5	95.6	98
252	N	K36R	9.10	23.7	30.3	24.3	13.3	46
469	N	E108D	86.60	77.0	63.9	51.8	84.7	51
1731	N	V529A	27.00	11.3	9.5	14.3	32.3	4
1769	N	L542L	26.20	9.9	9.0	14.1	31.8	4
1793	N	Ter550Q	74.20	50.1	45.8	43.6	62.6	44
1815	N	L557P	26.50	10.3	9.1	14.8	31.5	4
2145	V/P/I	N56T	40.50	44.2	40.1	31.8	31.5	38
3993	M	C244R	97.50	95.2	95.9	79.3	99.6	96
4822	F	P93S	45.30	41.4	38.4	30.8	31.1	38
5429/30	F	F295S	27.3	11.6	10.3	17.6	34.9	4
8318	HN	E569K	96.90	95.5	96.1	79.8	99.5	96
9369	L	D311G	43.40	33.4	36.8	29.1	29.9	38

p.i. post-infection

Table 17: Mutation frequencies identified by deep sequencing of RIT 4385.

Mutation frequencies of unpassaged virus and Vero and MDCK cell lysates 3 days post-infection (in duplicate).

Nucleotide position	Protein	Amino acid substitution	Mutation frequency (%)				
			Unpassaged	Vero		MDCK	
92	-	Leader	n.d.	n.d.	n.d.	n.d.	n.d.
252	N	K36R	n.d.	n.d.	n.d.	n.d.	n.d.
469	N	E108D	n.d.	n.d.	n.d.	n.d.	n.d.
1731	N	V529A	6.20	14.3	8.8	2.9	2.8
1769	N	L542L	16.60	19.9	23.1	28.7	34.4
1793	N	Ter550Q	n.d.	n.d.	n.d.	n.d.	n.d.
1815	N	L557P	21.30	31.5	30.0	30.4	35.7
2145	V/P/I	N56T	n.d.	n.d.	n.d.	n.d.	n.d.
3993	M	C244R	n.d.	n.d.	n.d.	n.d.	n.d.
4822	F	P93S	n.d.	n.d.	n.d.	n.d.	n.d.
5429/30	F	F295S	n.d.	n.d.	n.d.	n.d.	n.d.
8318	HN	E569K	n.d.	n.d.	n.d.	n.d.	n.d.
9369	L	D311G	n.d.	n.d.	n.d.	n.d.	n.d.

n.d. not detected, p.i. post-infection

5.4 Deep sequencing of JL-CK after growth in the neonatal rat brain

To investigate the population dynamics *in vivo*, amplicon-based deep sequencing was performed on RNA extracted from three rat brains, 4 days post-inoculation with JL-CK virus. Brains from rats inoculated with the RIT 4385 virus did not contain enough progeny virus to enable sufficient amplification for deep sequencing.

The frequencies of mutations in each brain compared to unpassaged JL-CK virus are shown in Table 18. The results obtained from each animal were largely consistent, although the difference in one animal (Brain 1) was less prominent, compared to the other two animals. Compared with the inoculate (JL-CK) or the JL-CK plaques (Table 15), the frequencies of the majority of the mutations decreased (E108D, V529A, L542L, Ter550Q, L557P, N56T, P93S, F295S and D311G).

The frequencies of the non-coding leader (g/a^{nt92}), C244R and E569K, which are present in all JL-CK variants, remained mostly unchanged. However, the K36R mutation in the nucleoprotein again was significantly more prominent after growth of the JL-CK virus in the rat brains. An observed 9.6 fold increase suggested that only the JL-CK1 virus had grown in the rat brain. All other mutations decreased in frequency, with the mutations unique to the JL-CK4 variant still present but at lower frequencies than observed after growth in Vero cells (3.5 fold decrease) or in unpassaged JL-CK virus (3.7 fold decrease). The other mutations were reduced in frequency to undetectable in some instances.

These results highlighted that the growth of the JL-CK virus in an *in vivo* system also alters the virus population dynamics of the vaccine, preferentially selecting the JL-CK1 variant.

Table 18: Comparison of mutation frequencies identified by deep sequencing of neonatal rat brains after inoculation with JL-CK virus.

Nucleotide position	Protein	Amino acid substitution	Mutation frequency (%)			
			Unpassaged	Brain 1	Brain 2	Brain 3
92	-	Leader	96.70	100	100	100
252	N	K36R	9.10	69.60	93.90	94.30
469	N	E108D	86.60	33.40	5.40	6.60
1731	N	V529A	27.00	1.30	n.d.	n.d.
1769	N	L542L	26.20	1.30	n.d.	n.d.
1793	N	Ter550Q	74.20	26.60	18.40	3.10
1815	N	L557P	26.50	1.20	n.d.	n.d.
2145	V/P/I	N56T	40.50	25.70	5.10	3.00
3993	M	C244R	97.50	99.90	100	100
4822	F	P93S	45.30	27.20	5.80	3.80
5429/30	F	F295S	27.3	1.2	n.d.	0.3
8318	HN	E569K	96.90	99.90	100	99.90
9369	L	D311G	43.40	26.00	5.50	3.50

n.d. not detected

The JL-CK vaccine population was not stable when passaged *in vivo* or in Vero cells with preferential selection of the JL-CK1 variant in both cases. The JL-CK4 variant only appeared to be selected for in Vero cells, but not in the rat brains. The growth of all other JL-CK variants was not supported in either model. By contrast, the JL-CK virus population remained stable in MDCK cells. These findings also highlighted the ability of MuV to adapt to cell lines and the speed at which MuVs can adapt.

CHAPTER 6 – DISCUSSION

Since the 1960's, live attenuated mumps vaccines have been produced in cell culture systems worldwide, yet the effects of the cell substrates on the genetics and biology of the resulting virus populations are poorly understood. MuV is a RNA virus and therefore has an intrinsic genetic instability due to the high error rate and lack of proof-reading of the RNA-dependant polymerase (Holland et al., 1982; Kenney et al., 2011). This leads to a diverse population of mutants, which are referred to as a quasispecies (Domingo et al., 1996). The ability of MuV to adapt to different cell substrates, including CEFs (Beck et al., 1989), canine kidney cells (Fedova et al., 1987), guinea pig kidney primary cells (Odisseev and Gacheva, 1994) and human diploid cells (Sassani et al., 1991), is a consequence of its quasispecies nature. It is this adaptation to cell substrates that forms the basis for the attenuation of MuV for vaccine production. However, the exact mechanism of attenuation of MuV is unknown, to date there are also no known genetic marker for attenuation (Boriskin Yu et al., 1988; Santak et al., 2014; Sauder et al., 2011; Shah et al., 2009).

It is recognised that different growth conditions and growth substrates can alter the ratio of Jeryl Lynn mumps subpopulations (Amexis et al., 2001; Amexis et al., 2002). It has also been documented that Vero cells can introduce mutations throughout the genome of MuV (Parker, 2013) and that serial passage of MuV through Vero cells accumulates defective interfering particles, that have been shown to attenuate a neurovirulence phenotype (Santak et al., 2014). The initial indication of a difference between the two vaccine viruses JL-CK and RIT 4385, described in this thesis, was based on the observation that they displayed different plaque morphologies in Vero cells, with JL-CK presenting a smaller plaque phenotype. However, the growth profile of both JL-CK and RIT 4385 in Vero cells was similar despite

JL-CK yielding a significantly higher progeny virus titre compared to RIT 4385 at similar timepoints. A difference in progeny virus yield was also observed after growth of JL-CK and RIT 4385 in MDCK cells, with JL-CK again producing significantly more progeny virus at both three and seven days post-infection. This result was not unexpected as the production of JL-CK on primary canine kidney cells is likely to have led to the virus adapting to canine kidney cells (Starke and Hlinak, 1974). It would have been interesting to compare progeny virus titres and plaque morphologies of JL-CK and RIT 4385 in primary canine kidney cells, had they been available and also to passage RIT 4385 through the primary cells to see if RIT 4385 could display a JL-CK-like phenotype. Similarly, it would have been interesting to perform passage through CEFs and examine progeny virus titres and plaque morphologies had they also been more freely available.

Neurovirulence testing of attenuated mumps seed viruses for use in vaccine manufacture is routinely performed in order to distinguish suitable attenuation of the MuV, using non-human primates. However, the neonatal rat model has been proposed as a more sensitive replacement (Rubin et al., 1998; Rubin et al., 2000). This model has been shown to distinguish between neurovirulent and non-neurovirulent MuVs (Rubin and Afzal, 2011; Rubin et al., 2005), with an increase in hydrocephalus which results in an enlarged ventricle correlating to an increase in neurovirulence potential. The neonatal rat model, however is complicated by the large amount of variability observed between animals, with large groups (30 - 60 animals) routinely used in order to obtain statistically significant results (Rubin et al., 1998; Rubin et al., 2000; Santak et al., 2014; Sauder et al., 2011).

In this study, the initial *in vivo* data suggested that JL-CK was more virulent than RIT 4385 in the neonatal rat model, causing a higher degree of hydrocephalus and producing higher titres of progeny virus in the brain. However, the degree of hydrocephalus observed for JL-CK

was not as severe as that seen in the neurovirulent wild-type Mu90. Despite these findings in the rats, there have been no reports of neurotropic adverse events occurring in patients who have received the JL-CK-containing vaccine, unlike patients who received the Urabe Am9 or Leningrad-3 vaccine strains (Cizman et al., 1989; Miller et al., 1993). The differences observed between JL-CK and RIT 4385 in the rat neurovirulence model here are however significant, as the differences between closely related strains described, have been hard to detect in previous studies (Rubin et al., 2005; Rubin et al., 2000). Viruses previously compared included the parent Jeryl Lynn vaccine consisting of two virus strains (JL-2 and JL-5), which was indistinguishable from RIT 4385 and the Urabe vaccine virus which was indistinguishable from a clinical isolate obtained from a Urabe vaccine-associated case of aseptic meningitis (Rubin et al., 2005). The differences observed between RIT 4385 and Mu90 in this thesis have been previously reported (Malik et al., 2009; Rubin et al., 2005).

The ferrets that were inoculated with JL-CK or RIT 4385 did not exhibit any clinical signs of MuV infection, which was in agreement with previously described studies of experimental infection of ferrets with MuV (Parker et al., 2013). However, there was a difference observed in the serum antibody responses of the ferrets inoculated with each virus, although the data must be interpreted with some caution due to the small number of animals used in this study. The ferrets inoculated with the JL-CK virus produced low levels of neutralising antibody, with neutralisation titres comparable to the pre-bleed samples. In contrast, neutralising antibodies were produced by ferrets in response to inoculation with RIT 4385, which had previously been shown to be mumps-specific (Parker et al., 2013). The neutralising antibody response from one ferret in particular showed a cross-protective effect by neutralising the Mu90 MuV. The human reference serum notably gave a low level of neutralising antibody ($ND_{50} = 12$), when challenged with the RIT 4385 virus. A neutralising

antibody level of 4 in human serum, when assessed by PRN, is generally considered to provide protection against mumps infection, (Mauldin et al., 2005). The low levels of neutralising antibody observed in ferrets inoculated with JL-CK may reflect the lower serological response previously observed in vaccinees receiving the JL-CK vaccine (Boxall et al., 2008; Lexova et al., 2013; Limberkova and Lexova, 2014; Mrazova et al., 2003; Plesnik et al., 1984). It would have been of interest to test the serum from patients who had been vaccinated with the JL-CK or RIT 4385 vaccines, however this material was unavailable for this study. Ferrets are not an established model for MuV infection (Parker et al., 2013) and the absence of a more suitable animal model for mumps limits the scope for testing neutralising antibody responses to infection.

The genomes of both JL-CK and RIT 4385 were compared by Sanger sequencing and aligned against the parental JL-5 database sequence to identify whether the differences observed could be attributed to changes at the genomic level. JL-CK displayed heterogeneity at thirteen positions throughout the genome compared to the parental JL-5 sequence and eleven positions compared to the RIT 4385 genome. Variations at these positions have not been reported before. This indicated that the JL-CK vaccine contained a higher level of heterogeneity than RIT 4385, when compared to the JL-5 sequence. The data obtained from MAPREC analysis of the N and P genes of JL-CK and RIT 4385, confirmed these polymorphic regions. Each of the genes throughout the JL-CK genome contained at least one polymorphic site, corresponding to predicted substitutions of encoded amino acids, and the non-coding leader sequence contained one mutation, g/a^{nt92}. The SH gene, which is the most variable (Cui et al., 2009; Takeuchi et al., 1991) and used to determine MuV genotype because of its variability (Afzal et al., 1997; Cui et al., 2009; Kunkel et al., 1995; Yeo et al., 1993), showed no genomic changes in JL-CK and had identical sequence to JL-5. The

majority of the variation observed was in the N gene with five polymorphic sites identified, one of which was a silent mutation (L542L). However the other mutations resulted in predicted amino acid changes (E108D, V529A, Ter550Q and L557P), with one in particular (Ter550Q) eliminating the Stop codon and extending the nucleoprotein by 21 amino acids. The extended nucleoprotein was shown to be expressed in JL-CK and mass spectrometry identified the presence of both the parent peptide (LTPIATIPGQSSHS) and the mutant peptide (PTPIATIPGQSSHS).

Isolation of viruses from individual plaques produced from JL-CK identified five JL-5 virus-variants within the JL-CK vaccine, plus a “Parental” virus which did not contain any of the polymorphic regions and was identical to the parent JL-5 strain. The five variants, which differed from the parent JL-5 virus, each contained the three mutations observed in the population; g/a^{nt92} in the non-coding leader sequence, C244R in the M protein and E569K in the HN protein, plus one or more of the other mutations that had been observed. Each of the JL-CK variants isolated from plaques were found at different frequencies as described in Table 14, with JL-CK1 and JL-CK4 identified at the highest frequencies of 46 % and 38 %, respectively. The remaining isolated variants were present at much lower frequencies; JL-CK2 at 7 %, JL-CK5 at 4 % and JL-CK3 at 2 % of the population, and the virus isolated which was identical to the JL-5 parent virus represented only 4 % of the population of the JL-CK vaccine. The frequencies of the JL-CK variants identified from plaques confirmed the earlier MAPREC data (Figure 19), with E108D, V529A, L542L and L557P each displaying band intensities in line with the frequencies of those mutations derived from the plaque data. The MAPREC signal intensities for mutations Ter550Q and N56T were lower than expected, considering that the variants containing those mutations are present in around 44 % (Ter550Q) and 38 % (N56T) of the JL-CK vaccine population (Table 14). This may be as a

consequence of the many variables associated with the MAPREC method. In addition, two of the polymorphisms in the N gene corresponding to L542L and L557P, were found in both JL-CK and RIT 4385 to a similar level by both MAPREC (Figure 19) and amplicon-based deep sequencing of the unpassaged virus (Table 15). Amplicon-based deep sequencing of JL-CK confirmed the presence of all of the mutations identified by Sanger sequencing of the isolated JL-CK plaques. In addition, the K36R mutation was identified in the JL-CK vaccine at low frequency (9.1 %), compared to the others, which may explain why it was not found initially by Sanger sequencing. Three of the heterogeneities (V529A, L542L and L557P) were also found in RIT 4385 at lower levels (6.2 %, 16.6 % and 21.3 % respectively), when RIT 4385 was analysed by deep sequencing. The V529A mutation had not been identified in RIT 4385 by Sanger sequencing or by MAPREC, this again is likely due to the low frequency of the mutation. Mutations, L542L and L557P were found to be present in RIT 4385 by MAPREC analysis, L542L is silent and L557P mutation is non-coding in this case.

Analysis of cell lysates three days post-infection with JL-CK showed that growth in Vero cells altered the frequencies of the mutations. The K36R mutation in the nucleoprotein of JL-CK1, increased three-fold during growth in Vero cells, even after only three days incubation, confirming previous findings that there is selective pressure from Vero cells on MuV (Afzal et al., 2005; Parker, 2013; Santak et al., 2014). Linkage between mutations cannot be confirmed by deep sequencing, due to the fragmentation step in the method. However, those mutations which were unique to a particular variant all behaved in a similar manner. The frequency of mutations associated with the JL-CK4 variant appeared to remain consistent, with N56T, P93S and D311G occurring at frequencies of 42.2 %, 39.9 % and 35 % in Vero lysates. This suggests that Vero cells preferentially select both JL-CK1 and JL-CK4. All other mutations reduced in frequency from unpassaged virus and were comparable to the plaque frequencies. The JL-CK variant population was less affected by passage on MDCK

cells, with the frequencies of mutations remaining comparable to the unpassaged JL-CK virus, suggesting a key effect from the species of cell used.

RIT 4385 was more genetically stable during growth in both Vero and MDCK cells. This may be because RIT 4385 appears to contain fewer virus variants compared to JL-CK. Plaque purification of RIT 4385 was not performed in order to isolate additional RIT 4385 variants, during this study. It is also possible that there are other regions of the RIT 4385 genome which are less stable after passage in cell lines, and have not been investigated in this study. These would have been identified if the whole genome of RIT 4385 had been analysed by shotgun sequencing however, this method was unable to be optimised for use during this study.

All of the JL-CK variants were capable of independent growth in Vero cells and all variants produced higher progeny virus titres than RIT 4385. In particular JL-CK1 produced the highest progeny virus titre in the shortest time (1.35×10^7 pfu/ml, 3 days p.i.) suggesting it may contain mutations that permit faster replication in Vero cells, which is in agreement with the deep sequencing data. In each of the JL-CK variants the nucleoprotein was the most variable, containing between one (JL-CK1) and four (JL-CK5) mutations. The K36R mutation was not identified in the JL-CK vaccine vial by Sanger sequencing, but was found to be present at low frequency (9.1 %) by amplicon-based deep sequencing and was shown to be present in the JL-CK1 variant, after Sanger sequencing of the isolated plaques. Variants JL-CK3, JL-CK4 and JL-CK5 all contain the Ter550Q mutation. The presence of the Ter550Q mutation in plaque isolates correlated with the small plaque phenotype. The nucleoprotein is required for transcription, replication and virus assembly (Ivancic-Jelecki et al., 2008). The first 375 amino acids of the nucleoprotein are required to form the nucleocapsid and modifications to the C terminal tail can affect assembly (Kingston et al.,

2004). During virus assembly, M proteins are thought to interact directly with the cytoplasmic tails of the F and HN glycoproteins and also with the nucleoprotein (Pei et al., 2010). Elongation of the nucleoprotein with or without the combination of the C244R mutation in the M protein, which is involved in virus assembly and budding from the cell membrane (Li et al., 2009), may affect the ability of progeny viruses to bud effectively from infected cells limiting the infection of neighbouring cells. The small plaque phenotype however was not the result of a growth defect, as the variants were all capable of growing in Vero cells to maximum comparable titres at day 4 post-infection, JL-CK3 1.6×10^6 pfu per ml, JL-CK4 2.3×10^6 pfu per ml and JL-CK5 1.4×10^6 pfu per ml. This result contradicts the deep sequencing results for the analysis of the cell lysates, 3 days post-infection, where JL-CK3 and JL-CK5 did not appear to grow well in Vero cells. This might be as a result of the further growth of the progeny viruses in Vero cells (over 10 days), to obtain the viral titre for each variant. Deep sequencing of cell lysates and Vero cell titres are therefore not a directly comparable method to identify whether specific viruses can grow in a cell line. It would be of interest to sequence the progeny virus plaques from the JL-CK3, JL-CK4 and JL-CK5 variants, to identify any change in the mutation frequency in comparison to the 3 day post-infection cell lysates.

None of the ferrets inoculated with the JL-CK variants showed any clinical signs of disease, which was also the case during the initial study where JL-CK and RIT 4385 were inoculated. There was considerable variation in neutralising antibody titre between each group of ferrets and also between each ferret in some cases. However, overall the sera from ferrets inoculated with RIT 4385, JL-CK1 and JL-CK3 produced an increase in serum antibody response by day 28, when challenged with the RIT 4385 virus. The serum antibody response to RIT 4385 in the two ferret studies was variable. This is most likely due to a difference in the stock

virus concentration used to prepare the inoculum, which was found to be lower than expected in the second study.

The sera from ferrets inoculated with JL-CK5 and from two of the ferrets inoculated with JL-CK4 demonstrated a maximum serum antibody response to the RIT 4385 challenge virus at day 7, which was similar to the response of the “Parental” virus isolated from JL-CK. In these cases, the neutralising antibody level began to reduce at day 14 and declined to the level of the pre-bleed sample by day 28. In these cases it is unlikely that the increase in ND₅₀ value at day 7 is due to neutralising antibody, this is more likely a response to cellular debris in the inocula which was remaining after isolation and growth of the variants in Vero cells, both the “Parental” and JL-CK5 viruses had undergone one additional passage in Vero cells, compared to the other variants. The Vero cell lysates had been centrifuged to remove cellular debris but the preparation is unlikely to be as pure as the vaccine preparation for RIT 4385 or JL-CK. It also is possible that any neutralisation response observed would be lower due to the challenge virus (RIT 4385) used. The sera from the ferrets inoculated with JL-CK4 and JL-CK5 may not have been able to cross neutralise the RIT 4385 virus. A cross-protective effect was suggested in the initial ferret study when the RIT 4385 vaccine was inoculated, however the sera from JL-CK inoculated ferrets did not appear to show cross-protection against the other challenge MuVs. Ideally, the challenge virus would have been the same as the virus used to inoculate each group, as in the initial study. However, there were limited stocks of each of the JL-CK variants and therefore to use these as the challenge virus in the PRN assays would have required a further passage in Vero cells, which may have introduced additional mutations. The observation that the ferrets inoculated with the “Parental” virus isolated from the JL-CK vaccine displayed a different neutralising antibody profile to RIT 4385 was unexpected. The “Parental” virus had undergone one additional passage in Vero cells, prior to inoculation into the ferrets (as had the JL-CK5 variant), to enable a virus stock

with a suitable titre to be used in the assay. In each case the virus had been sequenced over the known variable regions to ensure the mutations were still present (or not in the case of the “Parental” virus). However, it is not inconceivable that as the entire genomes of the “Parental” and JL-CK5 viruses were not sequenced there may have been the introduction of additional mutations at other sites which were undetected, with unknown effect(s) on the viruses.

The outcome of the ferret studies are further complicated in that there is no definitive correlate of protection in terms of neutralisation antibody for MuV. This makes it difficult to determine the level at which an ND₅₀ value has a biological meaning with regards to protective levels of antibody. The human reference serum control gave a neutralising antibody level of 12 which was lower than the day 28 values for the sera from RIT 4385, JL-CK1 and JL-CK3 inoculated ferrets (Figures 27 and 28). It has been reported that serum antibody levels as low as 2 and 4 can be considered protective in human serum (Ennis, 1969; Mauldin et al., 2005).

There have also been some concerns over the use of the PRN assay to determine neutralising ability and its correlation to protection. It has been shown that different virus strains and even the cell type used during the assay can affect the ND₅₀ value (Mukherjee et al., 2014; Pipkin et al., 1999). That, coupled with the lack of a suitable animal model for MuV infection and the findings that the ferret may not be the most suitable model for assessing neutralising antibodies against MuV (Parker et al., 2013) limits the biological conclusions from the ferret study.

There was a range of hydrocephalus severity observed in the rat brains after infection with the JL-CK variants and there was also variation between animals in each group. The hydrocephalus associated with JL-CK2 was significantly lower than RIT 4385 and the

hydrocephalus associated with JL-CK1 and JL-CK5 was significantly higher than RIT 4385 and comparable to JL-CK. Interestingly, the two variants which caused similar levels of hydrocephalus in the neonatal rat compared to JL-CK contained either the fewest mutations (JL-CK1) or the most mutations (JL-CK5). This suggests that the neurovirulence phenotype observed is not specific to one mutation. Therefore, there must be a requirement for a combination of mutations for a neurovirulence phenotype to be displayed. The neurovirulent Urabe MuV vaccine contains a mixture of two viruses which contain either a glutamic acid (E) or lysine (K) at position 335 of the HN protein. The virus containing K335 has been shown to be responsible for an increase in development of meningitis post-vaccination (Brown et al., 1996) and it has been shown that K335 changes the receptor binding and neuraminidase activity of the virus (Reyes-Leyva et al., 2007). However, studies involving recombinant Urabe MuV have identified that K335 is not responsible for a neurovirulence phenotype (Sauder et al., 2009). It has been demonstrated using recombinant MuV that combinations of genes can alter the neurovirulence phenotype observed in neonatal rats (Lemon et al., 2007; Sauder et al., 2011). Sauder et al. investigated gene-specific neurovirulence phenotypes showing that the Jeryl Lynn N and M genes in combination, could neuroattenuate a recombinant wild-type MuV. However, no combination of wild-type MuV genes could transfer a neurovirulent phenotype to a recombinant Jeryl Lynn MuV (Sauder et al., 2011). In contrast, a study by Lemon et al. showed the induction of a neurovirulence phenotype in a recombinant Jeryl Lynn MuV by insertion of the F and HN genes of a neurovirulent rodent-brain-adapted MuV, either individually or in combination (Lemon et al., 2007).

A correlation between neurovirulence potential and viral titre in the rat brain has been previously suggested (Sauder et al., 2011). However, comparison of the titres of the JL-CK variants in the brains of inoculated rats provided conflicting results when taken together with

the neurovirulence data, The JL-CK1 variant produced the highest progeny virus titres in the rat brain (3.5×10^4 pfu per ml, 4 days p.i.), which is in agreement with the neurovirulence data and amplicon-based deep sequencing of the neonatal rat brains which had been inoculated with JL-CK confirmed that the JL-CK1 variant was preferentially selected for in the rat brain. However, the maximum titres of the JL-CK5 variant (6.6×10^3 pfu per ml, 7 days p.i.) and JL-CK virus (2.5×10^3 pfu per ml, 10 days p.i.), were lower than that of the JL-CK1 variant despite displaying a similar neurovirulence potential. This could be due in part to the highly variable nature of the neonatal rat model and the fact that in these studies comparatively small numbers of animal were used in each group. Further studies would be useful to confirm these findings, which could not be initiated during the course of this study. The identification of a neurovirulence marker for MuV is lacking and appears to be a more complex issue than the identification of mutation(s). It has been described that changes in viral genetic heterogeneity may have an impact on MuV neurovirulence potential (Sauder et al., 2006). Amplicon-based deep sequencing of neonatal rat brains which had been inoculated with JL-CK also identified a dramatic change in the population of the JL-CK variants. The K36R mutation increased ten-fold and along with the non-coding leader, C244R and E569K mutations which are all found in the JL-CK1 variant were present at almost 100 % frequency, suggesting preferential selection of the JL-CK1 variant, which is in agreement with the high titre of the JL-CK1 variant observed. This may imply a role for these mutations in the neurovirulence phenotype. The other mutations all reduced in frequency, in particular mutations V529A, L542L, L557P and F295S, which are all unique to JL-CK5 were detected at only 1 % frequency or less.

The results for the neurovirulence studies and the deep sequencing of the rat brains provide conflicting data, with the JL-CK5 virus giving a high neurovirulence score but the deep sequencing suggesting that this virus does not replicate as well as the other JL-CK variants in

the rat brain. This could be due to the small number animal used in the neurovirulence studies and also in the analysis by deep sequencing, which was due to time constraints. A follow up study could provide valuable information on the growth of the JL-CK1 and JL-CK5 variants in the rat brain and the resulting neurovirulence phenotype could provide useful information on the role of the K36R, C244R and E569K mutations as a possible marker for neurovirulence.

The deep sequencing analysis also highlighted the variation found between animals infected with the same virus, with mutation frequencies displaying the same trend between animals but to varying degrees. In particular, the Ter550Q mutation (common to JL-CK3, JL-CK4 and JL-CK5) was found to decrease in frequency compared to the JL-CK virus (74.2 %). In each of the animals the frequencies were substantially different, RNV1 26.6 %, RNV2 18.4 % and RNV3 3.1 %. This may reflect a degree of preferential replication of JL-CK4 over JL-CK3 and JL-CK5 within the rat brain, which would be similar to the observation in Vero cells. This may explain the variation seen within groups of animals in the neurovirulence study. It would be of interest to confirm this finding in future studies with a larger group of animals and in combination with a neurovirulence study. This would provide valuable information linking an increase in ventricle size to a particular variant.

CONCLUSIONS

The two mumps viruses that were studied are closely related, originating from the same Jeryl Lynn parent strain. They differ only in their passage history, with JL-CK produced by passage in primary canine kidney cells and RIT 4385 produced by passage in CEFs. The aim of this project was to compare the biological characteristics of the JL-CK and RIT 4385 vaccine viruses *in vitro* and *in vivo*, identify any differences at the genomic level and examine how these genomic differences affect the biological properties of the JL-CK vaccine virus.

The data presented in this thesis confirmed the vaccine manufacturers' statements that both JL-CK and RIT 4385 comprise only the JL-5 virus strain. However, both viruses differ at the genomic level and in their biological phenotypes and suggested the JL-CK vaccine virus contained a more mixed population of MuVs compared to the RIT 4385 virus, which was previously unknown. The genetic differences between JL-CK and RIT 4385 are presumably a reflection of the passage history of the viruses in primary canine kidney cells and chick embryo fibroblasts respectively, although due to the lack of availability of primary canine kidney cells and CEF cells this was not assessed directly in this study. Further investigation revealed that the JL-CK vaccine virus contained a mixture of five JL-5 virus variants, plus the parent virus which has an identical sequence to the database sequence of the JL-5 virus.

The passage of the JL-CK virus, whether *in vitro* or *in vivo*, affects the genotype of the virus-variants and dramatically alters the composition of the virus population, within a short time, preferentially selecting some virus populations over others, JL-CK1 and JL-CK4 in Vero cells and JL-CK1 *in vivo*. The selection effects of the human host on the virus are unknown.

However, genomic variations identified in clinical MuV isolates following immunisation with the L-Zagreb vaccine strain have previously been reported (Gilliland et al., 2013).

The work covered in this thesis confirms the genetic instability of MuV in cell substrates and suggests that propagation of MuV on a cell substrate which is related to the passage history of the virus is likely to produce progeny virus which are more stable e.g. JL-CK growth in MDCK cells. It would be interesting to see if the same were true for RIT 4385 after growth in CEFs and clinical MuV isolates in a human cell line, for example *ex vivo* human airway epithelial cells as reported previously (Parker, 2013). At present the preferred cell line for plaque formation and titration of MuV is the Vero cell line. This study has shown that there is a selection pressure on the MuV in Vero cells and there may be some virus variants which do not produce plaques, which is of importance when determining MuV titres and potencies of vaccines for example. During the production of mumps vaccines, different batches are not routinely checked for mutations that may be introduced during growth. The findings from this thesis emphasise the importance of choosing suitable cell substrates for mumps vaccine manufacture.

The functions of the mutations identified were not assessed in this project and the use of a reverse genetics system to study the effects of particular mutations would be of interest. However, careful consideration of the cell lines used to produce recombinant MuVs would be required, to minimise the risk of introducing unwanted mutations. The use of a human derived cell line would be advantageous, in particular Hep2C (human cervical carcinoma), MCF-7 (human breast carcinoma) or HGT-1 (human stomach carcinoma), which have been shown to produce high titre, genetically stable progeny virus (Parker, 2013). Any recombinant viruses generated would need to be thoroughly characterised by deep

sequencing to ensure that no other genetic changes have occurred and that a single population of MuV was recovered.

The data from the neonatal rats provided conflicting data and there was considerable variation within experimental groups. This may in part be due to the small numbers of animal used for the studies. By combining the neurovirulence testing with deep sequencing, larger groups of animals could be used in future. Half of the brain could be fixed for neurovirulence scoring and the other half could be used for deep sequencing. This could provide a link to increased hydrocephalus and growth of a single population of MuV.

This study has generated valuable data which will be of interest to researchers in the paramyxovirus field. It has enabled the identification of mutations in the MuV genome which have not previously been described. These mutations are not known to be within any known antigenic or structurally relevant areas (inferred from related viruses). This study has also confirmed the previous findings that growth of MuV *in vitro* and *in vivo* alter the virus population dynamics and highlights that the changes to the viral population occur within three to four days of infection. The project has however also highlighted that there is still a lack of fundamental knowledge surrounding MuV. Much of what is known about MuV protein structure and function is inferred from related paramyxoviruses. The fact that still very little is known about MuV pathogenesis and the lack of a suitable animal model are limiting factors for future mumps pathogenesis research.

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APPENDICES

Appendix 1: Alignments of JL-CK and JL-5 protein sequences

JL-CK and JL-5 nucleoprotein amino acid sequence.

JL-CK	MSSVLKA ~~FER~~ F ~~T~~ IEQELQDRGEEGSIPPETLKS ~~AV~~ R VFVINTPNPTTRYQMLNFC ~~L~~ RIIC	60
JL-5	MSSVLKA ~~FER~~ F ~~T~~ IEQELQDRGEEGSIPPETLKS ~~AV~~ K VFVINTPNPTTRYQMLNFC ~~L~~ RIIC	60

JL-CK	SQNARASHRVGALITLFSLSAGMQNHIRLADRSP ~~EAQ~~ IERCEIDG F DPGTYRLIPNARA	120
JL-5	SQNARASHRVGALITLFSLSAGMQNHIRLADRSP ~~EAQ~~ IERCEIDG F EPGTYRLIPNARA	120

JL-CK	NLTANEIAAYALLADDLPPTINNGTPYVHADVEGQPCDEIEQFLDRCYSVLIQAWVMVCK	180
JL-5	NLTANEIAAYALLADDLPPTINNGTPYVHADVEGQPCDEIEQFLDRCYSVLIQAWVMVCK	180

JL-CK	CMTAYDQPAGSADRRFAKYQQQGRLEARYMLQPEAQRLIQTAIRKSLVVRQYLTFELQLA	240
JL-5	CMTAYDQPAGSADRRFAKYQQQGRLEARYMLQPEAQRLIQTAIRKSLVVRQYLTFELQLA	240

JL-CK	RRQGLLSNRYYAMVGDIGKYIENSGLTAF ~~FL~~ TLKYALG ~~TK~~ WSPLSLAAFTGELTKLRSLM	300
JL-5	RRQGLLSNRYYAMVGDIGKYIENSGLTAF ~~FL~~ TLKYALG ~~TK~~ WSPLSLAAFTGELTKLRSLM	300

JL-CK	MLYRGLGEQARYLALLEAPQIMDFAPGGYPLIFSYAMGVGTVL ~~DV~~ QMRNYTYARPFLNGY	360
JL-5	MLYRGLGEQARYLALLEAPQIMDFAPGGYPLIFSYAMGVGTVL ~~DV~~ QMRNYTYARPFLNGY	360

JL-CK	YFQIGVETARRQQGTVDNRVADDLGLTPEQRTEVTQLVDRLARGRGAGIPGGPVNPFVPP	420
JL-5	YFQIGVETARRQQGTVDNRVADDLGLTPEQRTEVTQLVDRLARGRGAGIPGGPVNPFVPP	420

JL-CK	VQQQQPAAVYEDIPALEESDDDGDEDGGAGFQNGVQLPAVRQGGQTD ~~FRA~~ QPLQDPIQ ~~AAQ~~	480
JL-5	VQQQQPAAVYEDIPALEESDDDGDEDGGAGFQNGVQLPAVRQGGQTD ~~FRA~~ QPLQDPIQ ~~AAQ~~	480

JL-CK	LFEMPLYPQVSNMPNNQNHQINRIGGLEHQDLLRYNENGDSQQDARGEH A NTFPNNPNQNA	540
JL-5	LFEMPLYPQVSNMPNNQNHQINRIGGLEHQDLLRYNENGDSQQDARGEH V NTFPNNPNQNA	540

JL-CK	QLQVGDWDE Q ITDMIKPTPIATIPGQSSHS	570
JL-5	QLQVGDWDE - -----	549

Comparison of JL-CK and JL-5 (accession AF201473). Amino acid substitutions; K36R, E108D, V529A, Ter550Q) are highlighted in **RED**, silent mutation (L542L) in **BOLD** font and the extension to the nucleoprotein is highlighted.

JL-CK and JL-5 phosphoprotein amino acid sequence.

JL-CK	MDQFIKQDETGDLIETGMNVANHFLSTPIQGTNSLSKASILPGVAPVLIGNPEQK T IQHP	60
JL-5	MDQFIKQDETGDLIETGMNVANHFLSTPIQGTNSLSKASILPGVAPVLIGNPEQK N IQHP	60

JL-CK	TASHQGSKTGRGSGVRSIIVSPSEAGNGGTQIPEPLFAQTGQGGIVTTVYQDPTIQPTG	120
JL-5	TASHQGSKTGRGSGVRSIIVSPSEAGNGGTQIPEPLFAQTGQGGIVTTVYQDPTIQPTG	120

JL-CK	SYRSVELAKIGKERMINRFVEKPRTSTPVTEFKRGAGSGCSRPNPRGGHRREWSLSWVQ	180
JL-5	SYRSVELAKIGKERMINRFVEKPRTSTPVTEFKRGAGSGCSRPNPRGGHRREWSLSWVQ	180

JL-CK	GEVRVFEWCNPICSPITAAARFHSCKCGNCPAKCDQCERDYGPP	224
JL-5	GEVRVFEWCNPICSPITAAARFHSCKCGNCPAKCDQCERDYGPP	224

Comparison of JL-CK and JL-5 (accession AF201473). Amino acid substitution N56T highlighted in **RED**

JL-CK and JL-5 matrix protein amino acid sequence.

JL-CK	MAGSQIKIPLPKPPDSDSQRLNAFFVIMAEQEGKGRLLRQIRLRKILSGDPSDQQITFVNT	60
JL-5	MAGSQIKIPLPKPPDSDSQRLNAFFVIMAEQEGKGRLLRQIRLRKILSGDPSDQQITFVNT	60

JL-CK	YGFIRATPETSEFISESSQQKVTPVVTACMLSFGAGPVLEDPQHMLKALDQTDIRVRKTA	120
JL-5	YGFIRATPETSEFISESSQQKVTPVVTACMLSFGAGPVLEDPQHMLKALDQTDIRVRKTA	120

JL-CK	SDKEQILFEINRIPNLFRRHYQISADHLIQASSDKYVKSPAKLIAGVNYIYCVTFLSVTVC	180
JL-5	SDKEQILFEINRIPNLFRRHYQISADHLIQASSDKYVKSPAKLIAGVNYIYCVTFLSVTVC	180

JL-CK	SASLKFRVARPPLAARSRLVRAVQMEILLRVTCCKDSQMAKSMLNDPDGEGCIASVWFHL	240
JL-5	SASLKFRVARPPLAARSRLVRAVQMEILLRVTCCKDSQMAKSMLNDPDGEGCIASVWFHL	240

JL-CK	CNL R KGRNKLRSYDENYFASKCRKMNLTVSIGDMWGPTILVHAGGHIPTTAKPFFNSRGW	300
JL-5	CNL C KGRNKLRSYDENYFASKCRKMNLTVSIGDMWGPTILVHAGGHIPTTAKPFFNSRGW	300
	*** *****	
JL-CK	VCHPIHQSSPSLAKTLWSSGCEIKAASAILQGS DYASLAKTDDIIYSKIKVDKDAANYKG	360
JL-5	VCHPIHQSSPSLAKTLWSSGCEIKAASAILQGS DYASLAKTDDIIYSKIKVDKDAANYKG	360

JL-CK	VSWSPFRKSASMRNL	375
JL-5	VSWSPFRKSASMRNL	375

Comparison of JL-CK and JL-5 (accession AF201473). Amino acid substitution (C244R) highlighted in **RED**

JL-CK and JL-5 fusion protein amino acid sequence.

JL-CK	MNAFPVICLGYAIFSSSICVNINTLQQIGYIKQQVRQLSYYSQSSSSYVVVKLLPNIQPT	60
JL-5	MNAFPVICLGYAIFSSSICVNINTLQQIGYIKQQVRQLSYYSQSSSSYVVVKLLPNIQPT	60

JL-CK	DNSCEFKSVTQYNKTLNLLLPIAENINNIAS S SLGSRRHKRFAGIAIGIAALGVATAAQ	120
JL-5	DNSCEFKSVTQYNKTLNLLLPIAENINNIAS P SLGSRRHKRFAGIAIGIAALGVATAAQ	120

JL-CK	VTAAVSLVQAQTNARAIAAMKNSIQATNRAVFEVKEGTQQLAIAVQAIQDHINTIMSTQL	180
JL-5	VTAAVSLVQAQTNARAIAAMKNSIQATNRAVFEVKEGTQQLAIAVQAIQDHINTIMSTQL	180

JL-CK	NNMSCQILDNQLATSLGLYLTELTTVFQPQLINPALSPISIQALRSLLGSMTPAVVQATL	240
JL-5	NNMSCQILDNQLATSLGLYLTELTTVFQPQLINPALSPISIQALRSLLGSMTPAVVQATL	240

JL-CK	STISISAAEILSAGLMEGQIVSVLLDEMOMIVKINIPTIVTQSNALVIDFYSSIS S INNQE	300
JL-5	STISISAAEILSAGLMEGQIVSVLLDEMOMIVKINIPTIVTQSNALVIDFYSSIS F INNQE	300

JL-CK	SIIQLPDRILEIGNEQWRYPAKNCKLTRHHMFCQYNEAERLSLETKLCLAGNISACVFSP	360
JL-5	SIIQLPDRILEIGNEQWRYPAKNCKLTRHHMFCQYNEAERLSLETKLCLAGNISACVFSP	360

JL-CK	IAGSYMRRFVALDGTIVANCRLTCLCKSPSYPIYQPDHHAHTTIDLTSCQTLSDGLDF	420
JL-5	IAGSYMRRFVALDGTIVANCRLTCLCKSPSYPIYQPDHHAHTTIDLTSCQTLSDGLDF	420

JL-CK	SIVSLSNITYTENLTISLSQTINTQPIDISTELSKVNASLQNAVKYIKESNHQLQSFSVG	480
JL-5	SIVSLSNITYTENLTISLSQTINTQPIDISTELSKVNASLQNAVKYIKESNHQLQSFSVG	480

JL-CK	SKIGAIIVSALVLSILSIIISLLFCCWAYIATKEIRRINFKTNHINTISSSVDDLIRY	538
JL-5	SKIGAIIVSALVLSILSIIISLLFCCWAYIATKEIRRINFKTNHINTISSSVDDLIRY	538

Comparison of JL-CK and JL-5 (accession AF201473). Amino acid substitutions (P93S and F295S) are highlighted in **RED**

JL-CK and JL-5 haemagglutinin-neuraminidase protein amino acid sequence.

JL-CK	MEPSKLFIMSDNATFAPGPFVNAAGKKTFRTCFRILVLSVQAVILILVIVTLGELIRMIN	60
JL-5	MEPSKLFIMSDNATFAPGPFVNAAGKKTFRTCFRILVLSVQAVILILVIVTLGELIRMIN	60

JL-CK	DQGLSNQLSSITDKIRESAAVIASAVGVMNQVIHGVTVSLPLQIEGNQNQLLSTLATICT	120
JL-5	DQGLSNQLSSITDKIRESAAVIASAVGVMNQVIHGVTVSLPLQIEGNQNQLLSTLATICT	120

JL-CK	NRNQVSNCSSTNIPLINDLRFINGINKFIIEDYATHDFSIGHPLNMPFSFIPTATSPNGCTR	180
JL-5	NRNQVSNCSSTNIPLINDLRFINGINKFIIEDYATHDFSIGHPLNMPFSFIPTATSPNGCTR	180

JL-CK	IPSFSLGKTHWCYTHNVINANCKDHTSSNQYVSMGILAQTAGYPMFKTLKIQYLS DGLN	240
JL-5	IPSFSLGKTHWCYTHNVINANCKDHTSSNQYVSMGILAQTAGYPMFKTLKIQYLS DGLN	240

JL-CK	RKSCSIATVPDGCAMYCYVSTQLETDDYAGSSPPTQKLILLFYNDTITERTISPSGLEGN	300
JL-5	RKSCSIATVPDGCAMYCYVSTQLETDDYAGSSPPTQKLILLFYNDTITERTISPSGLEGN	300

JL-CK	WATLVPGVGSGIYFENKLIFPAYGGVLPNSTLGVKLAREFFRPVNPYNPCSGPQQEELDQR	360
JL-5	WATLVPGVGSGIYFENKLIFPAYGGVLPNSTLGVKLAREFFRPVNPYNPCSGPQQEELDQR	360

JL-CK	ALRSYFPSYFSSRRVQSAFLVCAWNQILVTNCELVPSNNQTLMGAEGRVLLINNRLLYY	420
JL-5	ALRSYFPSYFSSRRVQSAFLVCAWNQILVTNCELVPSNNQTLMGAEGRVLLINNRLLYY	420

JL-CK	QRSTSWWPYELLYEISFTFTNYGQSSVNMSWIPIYSFTRPGSGHCSENVCPIVCVSGVY	480
JL-5	QRSTSWWPYELLYEISFTFTNYGQSSVNMSWIPIYSFTRPGSGHCSENVCPIVCVSGVY	480

JL-CK	LDPWPLTPYRHQSGINRNFYFTGALLNSSTTRVNPTLYVSALNNLKVLPAPYGTQGLFASY	540
JL-5	LDPWPLTPYRHQSGINRNFYFTGALLNSSTTRVNPTLYVSALNNLKVLPAPYGTQGLFASY	540

JL-CK	TTTTCFQDTGDASVYCVYIMELASNIVG K FQILPVLARLTIT	582
JL-5	TTTTCFQDTGDASVYCVYIMELASNIVG E FQILPVLARLTIT	582

Comparison of JL-CK and JL-5 (accession AF201473). Amino acid substitution (E569K) highlighted in **RED**

JL-CK and JL-5 large protein amino acid sequence.

JL-CK	MAGLNEILLPEVHLNSPIVRYKLFYYILHGQLPNDLEPDDLGPLANQNWKAIRAEESQVH	60
JL-5	MAGLNEILLPEVHLNSPIVRYKLFYYILHGQLPNDLEPDDLGPLANQNWKAIRAEESQVH *****	60
JL-CK	ARLKQIRVELIARIPSLRWTRSQREIAILLIWPRIIPILQAYDLRQSMQLPTVWEKLTQST	120
JL-5	ARLKQIRVELIARIPSLRWTRSQREIAILLIWPRIIPILQAYDLRQSMQLPTVWEKLTQST *****	120
JL-CK	VNLISDGLERVVHLHISNQLTGKPNLFTRSAGQDTKDYSIPSTRELSQIWFNNEWSGSVK	180
JL-5	VNLISDGLERVVHLHISNQLTGKPNLFTRSAGQDTKDYSIPSTRELSQIWFNNEWSGSVK *****	180
JL-CK	TWLMIKYRMRQLITNQKTGELTDLVTIVDTRSTLCIITPELVALYSSEHKALTYLTFEMV	240
JL-5	TWLMIKYRMRQLITNQKTGELTDLVTIVDTRSTLCIITPELVALYSSEHKALTYLTFEMV *****	240
JL-CK	LMVTDMLEGRNLNVSSLCTASHYLSPLKKRIEVLTLVDDLALLMGDKVYGIVSSLESFVY	300
JL-5	LMVTDMLEGRNLNVSSLCTASHYLSPLKKRIEVLTLVDDLALLMGDKVYGIVSSLESFVY *****	300
JL-CK	AQLQYGDVPVIGIKGTFYGFICNEILDLLTEDNIFTEEEANKVLLDLTSQFDNLSPDLTAE	360
JL-5	AQLQYGDVPVIDIKGTFYGFICNEILDLLTEDNIFTEEEANKVLLDLTSQFDNLSPDLTAE *****	360
JL-CK	LLCIMRLWGHPTLTASQAASKVRESMCAPKVLDFQTIMKTLAFFHAILINGYRRSHNGIW	420
JL-5	LLCIMRLWGHPTLTASQAASKVRESMCAPKVLDFQTIMKTLAFFHAILINGYRRSHNGIW *****	420
JL-CK	PPTTLHGNA PKSLIEMRHDNSELKYEYVLKNWKSISMLRIHKCFDASPDEDLSIFMKDKA	480
JL-5	PPTTLHGNA PKSLIEMRHDNSELKYEYVLKNWKSISMLRIHKCFDASPDEDLSIFMKDKA *****	480
JL-CK	ISCPQDWMGVFRRSLIKQRYRDANRPLPQPFNRRLLLNFLEDDRFDPIKELEYVTSGEY	540
JL-5	ISCPQDWMGVFRRSLIKQRYRDANRPLPQPFNRRLLLNFLEDDRFDPIKELEYVTSGEY *****	540
JL-CK	LRDPEFCASYSLKEKEIKATGRIFAKMTKMRSCQVIAESLLANHAGKLMRENGVVLDQL	600
JL-5	LRDPEFCASYSLKEKEIKATGRIFAKMTKMRSCQVIAESLLANHAGKLMRENGVVLDQL *****	600
JL-CK	KLTKSLLTMNQIGIISEHSRRSTADNMTLAHSGSNKHRINNSQFKKNKDNKHEMPDDGFE	660
JL-5	KLTKSLLTMNQIGIISEHSRRSTADNMTLAHSGSNKHRINNSQFKKNKDNKHEMPDDGFE *****	660
JL-CK	IAACFLTDDLTKYCLNWRYQVIIPFARTLNSMYGIPHLFEWIHLRLMRSTLYVGDPFNPP	720
JL-5	IAACFLTDDLTKYCLNWRYQVIIPFARTLNSMYGIPHLFEWIHLRLMRSTLYVGDPFNPP *****	720
JL-CK	SDPTQLDLDTALNDDIFIVSPRGGIEGLCQKLWTMISISTIIISATEANTRVMSMVQGDN	780
JL-5	SDPTQLDLDTALNDDIFIVSPRGGIEGLCQKLWTMISISTIIISATEANTRVMSMVQGDN *****	780
JL-CK	QAIAITTRVVRSLSHSEKKEQAYKASKLFFERLRANNHGIGHHLKEQETILSSDFFIYSK	840
JL-5	QAIAITTRVVRSLSHSEKKEQAYKASKLFFERLRANNHGIGHHLKEQETILSSDFFIYSK *****	840

JL-CK	RVFYKGRILTQALKNVSKMCLTADILGDCSQASCSNLATTVMRLTENGVEKDLCYFLNAF	900
JL-5	RVFYKGRILTQALKNVSKMCLTADILGDCSQASCSNLATTVMRLTENGVEKDLCYFLNAF	900

JL-CK	MTIRQLCYDLVFPQTKSLSDITNAYLNHPILISRLCLLPSQLGGLNFLSCSRLFNRNIG	960
JL-5	MTIRQLCYDLVFPQTKSLSDITNAYLNHPILISRLCLLPSQLGGLNFLSCSRLFNRNIG	960

JL-CK	DPLVSAIADV KRLIKAGCLDIWVLYNILGRRPGKGKWT LAADPYTLNIDYLPSTTFLK	1020
JL-5	DPLVSAIADV KRLIKAGCLDIWVLYNILGRRPGKGKWT LAADPYTLNIDYLPSTTFLK	1020

JL-CK	KHAQYTLMERSVNPMLRGVFSENAAAAEEEEELAQYLLDREVVMPRVAHVILAQSSCGRRKQ	1080
JL-5	KHAQYTLMERSVNPMLRGVFSENAAAAEEEEELAQYLLDREVVMPRVAHVILAQSSCGRRKQ	1080

JL-CK	IQGYLDSTRTIIRYSLEVRPLSAKKLNTVIEYNLLYLSYNLEIEKPNIVQPFLNAINVD	1140
JL-5	IQGYLDSTRTIIRYSLEVRPLSAKKLNTVIEYNLLYLSYNLEIEKPNIVQPFLNAINVD	1140

JL-CK	TCSIDIARSLRKLSWATLLNGRPIEGLETPDPIELVHGCLIIGSDECEHCSSGDDKFTWF	1200
JL-5	TCSIDIARSLRKLSWATLLNGRPIEGLETPDPIELVHGCLIIGSDECEHCSSGDDKFTWF	1200

JL-CK	FLPKGIRLDDDPASNPPPIRVPIYIGSKTDERRVASMAYIKGASVSLKSALRLAGVYIWAFF	1260
JL-5	FLPKGIRLDDDPASNPPPIRVPIYIGSKTDERRVASMAYIKGASVSLKSALRLAGVYIWAFF	1260

JL-CK	DTEESWQDAYELASTRVNLTLEQLQSLTPLPTSANLVHRLDDGTTQLKFTPASSYAFSSF	1320
JL-5	DTEESWQDAYELASTRVNLTLEQLQSLTPLPTSANLVHRLDDGTTQLKFTPASSYAFSSF	1320

JL-CK	VHISNDCQILEIDDDQVTDNLIYQQVMITGLALIIETWNNPPINF SVYETTLHLHTGSSCC	1380
JL-5	VHISNDCQILEIDDDQVTDNLIYQQVMITGLALIIETWNNPPINF SVYETTLHLHTGSSCC	1380

JL-CK	IRPVESCVVNPPLL PVPLINVPQMKNFVYDPEPLS LLEMEKIEDIAYQTRIGGLDQIPLL	1440
JL-5	IRPVESCVVNPPLL PVPLINVPQMKNFVYDPEPLS LLEMEKIEDIAYQTRIGGLDQIPLL	1440

JL-CK	EKIPLL AHLTAKQMVNSITGLDEATSIMNDAVVQADYTSNWISECCYTYIDS VFVYSGWA	1480
JL-5	EKIPLL AHLTAKQMVNSITGLDEATSIMNDAVVQADYTSNWISECCYTYIDS VFVYSGWA	1480

JL-CK	LLLELSYQMYYLRIQGIQGILDYVYMTLRRIPGMAITGISSTISHPRILRRCINLDVIAP	1500
JL-5	LLLELSYQMYYLRIQGIQGILDYVYMTLRRIPGMAITGISSTISHPRILRRCINLDVIAP	1500

JL-CK	INSPHIASLDYTKLSIDAVMWGTKQVLTNISQGIDYEIVVPSESQTLTSDRVNLNVARKL	1560
JL-5	INSPHIASLDYTKLSIDAVMWGTKQVLTNISQGIDYEIVVPSESQTLTSDRVNLNVARKL	1560

JL-CK	SLLAIIWANYNPPKVKGMSPEDKCQAL TTHLLQTVEYVEYIQIEKTNIRRMIEPKLTA	1620
JL-5	SLLAIIWANYNPPKVKGMSPEDKCQAL TTHLLQTVEYVEYIQIEKTNIRRMIEPKLTA	1620

JL-CK	YPSNLFYLSRKLLNAIRDSEEGQFLIASYYNSFGYLEPILMESKIFNLSSSESASLTEFD	1680
JL-5	YPSNLFYLSRKLLNAIRDSEEGQFLIASYYNSFGYLEPILMESKIFNLSSSESASLTEFD	1680

JL-CK	FILNLELSDASLEKYSLPSLLMTAENMDNPFQPPPLHHVLRPLGLSSTSWYKTISVLNYI	1740
JL-5	FILNLELSDASLEKYSLPSLLMTAENMDNPFQPPPLHHVLRPLGLSSTSWYKTISVLNYI	1740

JL-CK	SHMKISDGAHLYLAEGSGASMSLIETFLPGETIWYNSLFNSGENPPQRNFAPLPTQFIES	1800
JL-5	SHMKISDGAHLYLAEGSGASMSLIETFLPGETIWYNSLFNSGENPPQRNFAPLPTQFIES	1800

JL-CK	VPYRLIQAGIAAGNGIVQSFYPLWNGNSDITDLSTKTSVEYIIHKVGADTCALVHVDLEG	1860
JL-5	VPYRLIQAGIAAGNGIVQSFYPLWNGNSDITDLSTKTSVEYIIHKVGADTCALVHVDLEG	1860

JL-CK	VPGSMNSMLERAQVHALLITVTVLKPGGLLILKASWEPFNRFSFLLTVLWQFFSTIRILR	1920
JL-5	VPGSMNSMLERAQVHALLITVTVLKPGGLLILKASWEPFNRFSFLLTVLWQFFSTIRILR	1920

JL-CK	SSYSDPNNHEVYIIATLAVDPTTSSFTTALNRARTLNEQGFSLIPPELVSEYWRKRVEQG	1980
JL-5	SSYSDPNNHEVYIIATLAVDPTTSSFTTALNRARTLNEQGFSLIPPELVSEYWRKRVEQG	1980

JL-CK	QIIQDCIDKVISECVRDQYLADNNIILQAGGTPSTRKWLDLPDYSSFNELQSEMARLITI	2040
JL-5	QIIQDCIDKVISECVRDQYLADNNIILQAGGTPSTRKWLDLPDYSSFNELQSEMARLITI	2040

JL-CK	HLKEVIEILKGQASDHDTLTFTSYNVGPLGKINTILRLIVERILMYTVRNWCILPTQTRL	2100
JL-5	HLKEVIEILKGQASDHDTLTFTSYNVGPLGKINTILRLIVERILMYTVRNWCILPTQTRL	2100

JL-CK	TLRQSIELGEFRLRDVITPMEILKLSPNRKYLKSALNQSTFNHLMGETSDILLNRAYQKR	2160
JL-5	TLRQSIELGEFRLRDVITPMEILKLSPNRKYLKSALNQSTFNHLMGETSDILLNRAYQKR	2160

JL-CK	IWKAIGCVIYCFGLLTPDVEGSERIDVDNDIPDYDIHGDII	2201
JL-5	IWKAIGCVIYCFGLLTPDVEGSERIDVDNDIPDYDIHGDII	2201

Comparison of JL-CK and JL-5 (accession AF201473). Amino acid substitution (D311G) highlighted in **RED**

Appendix 2a: RIT 4385 nucleoprotein identification by LC-MS/MS

Native Jeryl Lynn nucleoprotein (UniProt Q77158)

MSSVLKA FER **FTIEQELQDR** **GEEGSIPPET** **LKSAVKVFVI** **NTPNPTRYQ** MLNFCLRIIC
 SQNARASHRV **GALITLFSLP** **SAGMQNHRL** **ADRSPEAQIE** RCEIDGFEPG TYRLIPNARA
 NLTANEIAAY ALLADDLPPT INNGTPYVHA DVEGQPCDEI EQFLDRCYSV LIQAWVMVCK
 CMTAYDQPAG SADRRFAKYQ QQGRLEARYM **LQPEAQR**LIQ TAIRKSLVVR **QYLTFELQLA**
RRQGLLSNRY **YAMVGDIGKY** **IENSGLTAF**F **LT**LK^YALGTK **WSPLSLAAFT** **GELTK**LRSLM
 MLYRGLGEQA RYLALLEAPQ IMDFAPGGYP LIFS^YAMGVG TVLDVQMRNY TYARPFLNGY
 YFQIGVETAR RQOGTVDNRV **ADDLGLTPEQ** **RTEVTQLVDR** LARGRGAGIP GGPVNPFPVPP
 VQQQQPAAVY EDIPALEESD DDGDEDGGAG FQNGVQLPAV RQGGQTDFA **QPLQDPIQAQ**
LFMPLYPQVS **NMPNNQNHQI** **NRIGGLEHQD** **LLR**YNENGDS QQDAR**GEHVN** **TFPNNPNQNA**
QLOVGDWDE

10 peptides (highlighted) were matched, corresponding to 214 / 549 residues (39 %) sequence coverage, which was identical to the MuV (Jeryl Lynn) nucleoprotein (UniProt Q77158).

The carboxy-terminal peptide was identified (**bold/underlined**).

Modified Jeryl Lynn nucleoprotein containing the carboxy-terminal putative additional amino acids QITDMIKPTPIATIPGQSSHS

MSSVLKA FER **FTIEQELQDR** **GEEGSIPPET** **LKSAVKVFVI** **NTPNPTRYQ** MLNFCLRIIC
 SQNARASHRV **GALITLFSLP** **SAGMQNHRL** **ADRSPEAQIE** RCEIDGFEPG TYRLIPNARA
 NLTANEIAAY ALLADDLPPT INNGTPYVHA DVEGQPCDEI EQFLDRCYSV LIQAWVMVCK
 CMTAYDQPAG SADRRFAKYQ QQGRLEARYM **LQPEAQR**LIQ TAIRKSLVVR **QYLTFELQLA**
RRQGLLSNRY **YAMVGDIGKY** **IENSGLTAF**F **LT**LK^YALGTK **WSPLSLAAFT** **GELTK**LRSLM
 MLYRGLGEQA RYLALLEAPQ IMDFAPGGYP LIFS^YAMGVG TVLDVQMRNY TYARPFLNGY
 YFQIGVETAR RQOGTVDNRV **ADDLGLTPEQ** **RTEVTQLVDR** LARGRGAGIP GGPVNPFPVPP
 VQQQQPAAVY EDIPALEESD DDGDEDGGAG FQNGVQLPAV RQGGQTDFA **QPLQDPIQAQ**
LFMPLYPQVS **NMPNNQNHQI** **NRIGGLEHQD** **LLR**YNENGDS QQDAR**GEHVN** **TFPNNPNQNA**
QLOVGDWDE***Q ITDMIKPTPI ATIPGQSSHS***

9 peptides (highlighted) were matched, corresponding to 214 / 570 (37.5 %) sequence coverage, against the modified MuV (Jeryl Lynn) nucleoprotein. *Italic* indicates the additional putative amino acids at the carboxy-terminal, including P557. No matching carboxy-terminal peptide (**underlined**) was identified.

Modified Jeryl Lynn nucleoprotein containing the carboxy-terminal putative additional amino acids QITDMIKLTPIATIPGQSSH

MSSVLKA FER **FTIEQELQDR** **GEEGSIPPET** **LKSAVKVFVI** **NTPNPTRYQ** MLNFCLRIIC
 SQNARASHRV **GALITLFSLP** **SAGMQNHRL** **ADRSPEAQIE** RCEIDGFEPG TYRLIPNARA
 NLTANEIAAY ALLADDLPPT INNGTPYVHA DVEGQPCDEI EQFLDRCYSV LIQAWVMVCK
 CMTAYDQPAG SADRRFAKYQ QQGRLEAR**YM** **LQPEAQR**LIQ TAIRKSLVVR **QYLT FELQLA**
RRQGLLSNRY **YAMVGDIGKY** **IENSGLTAF** **LTLKYALGTK** **WSPLSLAAFT** **GELTKLRSLM**
 MLYRGLGEQA RYLALLEAPQ IMDFAPGGYP LIFS YAMGVG TVLDVQMRNY TYARPFLNGY
 YFQIGVETAR RQQGTVDNR**V** **ADDLGLTPEQ** **RTEVTQLVDR** LARGRGAGIP GGPVNPFPVP
 VQQQQPAAVY EDIPALEESD DDGDEDGGAG FQNGVQLPAV RQGGQTD**FRA** **QPLQDPIQAQ**
LFMPLYPQVS **NMPNNQNHQI** **NRIGGLEHQD** **LLRYNENGDS** QQDARGEHVN TFPNNPNQNA
 QLQVGDWDE **Q ITDMIKLTPI ATIPGQSSH**

9 peptides (highlighted) were matched, corresponding to 214 / 570 (37.5 %) sequence coverage, against the modified MuV (Jeryl Lynn) nucleoprotein. *Italic* indicates the additional putative amino acids at the carboxy-terminal, including L557. No matching carboxy-terminal peptide (underlined) was identified.

Appendix 2b: JL-CK nucleoprotein identification by LC-MS/MS

Native Jeryl Lynn nucleoprotein (UniProt Q77158)

MSSVLKA FER **FTIEQELQDR** **GEEGSIPPET** **LKSAVKVFVI** **NTPNPTRYQ** MLNFCLRIIC
 SQNARASHRV **GALITLFLSLP** **SAGMQNHRL** **ADRSPEAQIE** RCEIDGFEPG TYRLIPNARA
 NLTANEIAAY ALLADDLPPT INNGTPYVHA DVEGQPCDEI EQFLDRCYSV LIQAWVMVCK
 CMTAYDQPAG SADRRFAKYQ QQGRLEARYM **LQPEAQR**LIQ TAIRKSLVVR **QYLTFELQLA**
RRQGLLSNRY **YAMVGDIGKY** **IENSGLTAF**F **LT**LK^YALGTK **WSPLSLAAFT** **GELTK**LRSLM
 MLYRGLGEQA RYLALLEAPQ IMDFAPGGYP LIFS^YAMGVG TVLDVQMRNY TYARPFLNGY
 YFQIGVETAR RQOGTVDNRV **ADDLGLTPEQ** **RTEVTQLVDR** LARGRGAGIP GGPVNPFPVP
 VQQQQPAAVY EDIPALEESD DDGDEDGGAG FQNGVQLPAV RQGGQTDFA **QPLQDPIQAQ**
LFMPLYPQVS **NMPNNQNHQI** **NRIGGLEHQD** **LLR**YNENGDS QQDARGEHVN TFPNNPNQNA
QLOVGDWDE

9 peptides (highlighted) were matched, corresponding to 190 / 549 residues (34.6 %) sequence coverage, which was identical to the MuV (Jeryl Lynn) nucleoprotein (UniProt Q77158). The carboxy-terminal peptide was not identified (underlined).

Modified Jeryl Lynn nucleoprotein containing the carboxy-terminal putative additional amino acids QITDMIKPTPIATIPGQSSHS

MSSVLKA FER **FTIEQELQDR** **GEEGSIPPET** **LKSAVKVFVI** **NTPNPTRYQ** MLNFCLRIIC
 SQNARASHRV **GALITLFLSLP** **SAGMQNHRL** **ADRSPEAQIE** RCEIDGFEPG TYRLIPNARA
 NLTANEIAAY ALLADDLPPT INNGTPYVHA DVEGQPCDEI EQFLDRCYSV LIQAWVMVCK
 CMTAYDQPAG SADRRFAKYQ QQGRLEARYM **LQPEAQR**LIQ TAIRKSLVVR **QYLTFELQLA**
RRQGLLSNRY **YAMVGDIGKY** **IENSGLTAF**F **LT**LK^YALGTK **WSPLSLAAFT** **GELTK**LRSLM
 MLYRGLGEQA RYLALLEAPQ IMDFAPGGYP LIFS^YAMGVG TVLDVQMRNY TYARPFLNGY
 YFQIGVETAR RQOGTVDNRV **ADDLGLTPEQ** **RTEVTQLVDR** LARGRGAGIP GGPVNPFPVP
 VQQQQPAAVY EDIPALEESD DDGDEDGGAG FQNGVQLPAV RQGGQTDFA **QPLQDPIQAQ**
LFMPLYPQVS **NMPNNQNHQI** **NRIGGLEHQD** **LLR**YNENGDS QQDARGEHVN TFPNNPNQNA
QLOVGDWDE***Q ITDMIKPTPI ATIPGQSSHS***

9 peptides (highlighted) were matched, corresponding to 190 / 570 residues (33.3 %) sequence coverage, against the modified MuV (Jeryl Lynn) nucleoprotein. *Italic* indicates the additional putative amino acids at the carboxy-terminal, including P557. No matching carboxy-terminal peptide (underlined) was identified.

Modified Jeryl Lynn nucleoprotein containing the carboxy-terminal putative additional amino acids QITDMIKLTPIATIPGQSSH

MSSVLKA FER **FTIEQELQDR** **GEEGSIPPET** **LKSAVKVFVI** **NTPNPTRYQ** MLNFCLRIIC
 SQNARASHRV **GALITLFSLP** **SAGMQNHRL** **ADRSPEAQIE** RCEIDGFEPG TYRLIPNARA
 NLTANEIAAY ALLADDLPPT INNGTPYVHA DVEGQPCDEI EQFLDRCYSV LIQAWVMVCK
 CMTAYDQPAG SADRRFAKYQ QQGRLEARYM **LQPEAQR**LIQ TAIRKSLVVR **QYLT FELQLA**
RRQGLLSNRY **YAMVGDIGKY** **IENSGLTAF** **LTLKYALGTK** **WSPLSLAAFT** **GELTKLRSLM**
 MLYRGLGEQA RYLALLEAPQ IMDFAPGGYP LIFS YAMGVG TVLDVQMRNY TYARPFLNGY
 YFQIGVETAR RQQGTVDNRV **ADDLGLTPEQ** **RTEVTQLVDR** LARGRGAGIP GGPVNPFVPP
 VQQQQPAAVY EDIPALEESD DDGDEDGGAG FQNGVQLPAV RQGGQTDFRA **QPLQDP IQAQ**
LFMPLYPQVS **NMPNNQNHQI** **NRIGGLEHQD** **LLRYNENGDS** QQDARGEHVN TFPNNPNQNA
 QLQVGDWDEQ ***ITDMIKLTPI ATIPGQSSH***

10 peptides (highlighted) were matched, corresponding to 203 / 570 residues (35.6 %) sequence coverage, against the modified MuV (Jeryl Lynn) nucleoprotein. *Italic* indicates the additional putative amino acids at the carboxy-terminal, including L557. The carboxy-terminal peptide (***ITDMIKLTPI ATIPGQSSH***) was identified.

Appendix 3: Publications

Connaughton, S. M., Wheeler, J.X., Vitková, E., Minor, P. and Schepelmann, S. (2015), “*In vitro* and *in vivo* growth alter the population dynamic and properties of a Jeryl Lynn mumps vaccine” *Vaccine*, **33**: 4586-4593.

Parker, L., Gilliland, S. M., Minor, P. and Schepelmann, S. (2013), “Assessment of the ferret as an *in vivo* model for mumps virus infection” *Journal of General Virology*, **94**: 1200-1205.

Gilliland, S. M., Jenkins, A., Parker, L., Somdach, N., Pattamadilok, S., Incomserb, P., Berry, N., Schepelmann, S. and Minor, P. (2013), “Vaccine-related mumps infections in Thailand and the identification of a novel mutation in the mumps fusion protein” *Biologicals*, **41**: 84-87.