Evaluation of a rat model for mumps virus neurovirulence testing

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Evaluation of a Rat Model for Mumps Virus Neurovirulence Testing

Caroline Louise Powell BSc (Hons)

A thesis submitted in partial fulfilment of the requirements of the Open University for the degree of Master of Philosophy

18th June 2004

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Abstract

Safety is an important aspect in the licensing of any vaccine. Prior to placement on the market, any risks and side-effects attributable to the vaccine must be as well documented as possible. One of the main priorities in the safety of a vaccine containing live microorganisms is the level of neurovirulent activity of the vaccine components. Every batch of the oral polio vaccine is tested for neurovirulent activity by inoculation into the central nervous system of macaques. Seed stocks for the live mumps virus vaccines are tested likewise. However, relying on the macaque model is an inadequate marker of neurovirulence of mumps vaccines as the animal system does not fully mimic the human pathological processes, thereby making results difficult to interpret. Further inadequacies of this model include the small experimental group used, the cost of purchasing and maintaining macaques, as well as the ethics involved in using non-human primates. As a result, many alternative animal models have been investigated, from using different monkey species to using smaller animals. Of the animal models investigated, the marmoset monkey model shows prospects; however, the use of large animals is not desirable. Of the smaller animal models, the rat model, currently being developed by the Food and Drug Administration in USA and NIBSC, holds many advantages. Rats are cheaper, easier to handle and can be used in larger experimental groups, and the CNS of neonatal rats shows recordable and reproducible susceptibility to the mumps virus. The aim of this project was to evaluate the rat model using various vaccine, vaccine-derived and wild-type viruses. These were used in order to establish if the model can distinguish between virulent wild-type and attenuated vaccine strains and mixed and pure preparations of vaccines of a related strain shown to have caused meningitis in children. The results conclude that the neo-natal rat model is on a par with the macaque model in distinguishing between wild-type and vaccine strains; however, neither model can distinguish between a vaccine and its derived variants causing adverse events in the recipients. However, a smaller animal model is more desirable and the method of diagnosing
neurovirulent activity in rats, by measuring the enlargement of the lateral ventricle, is much simpler than histological analysis of lesions in the macaque brain. The results of the FDA/NIBSC collaboration showed varying degrees of difference, highlighting a need to standardize the test for future applications.
For Mum and Dad
Acknowledgements

I wish to acknowledge the help and support of Drs Muhammed Afzal, Jim Robertson and Phil Minor.

Special thanks to Maureen Bentley for all her help and work on this project, which couldn’t have been done without her.

Many thanks to Steve Crane for teaching me the finer aspects of histology and Radio London, and to Ghazi Auda for teaching me analysis of histology samples.

A big thank you to the veterinarians and animal technicians for inoculating and looking after the rat pups.

Big hugs and thank you to my friends who have had to endure my endless complaining and moaning. Thanks for making me an alcoholic!!
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<td>Complementary DNA</td>
</tr>
<tr>
<td>CEF</td>
<td>Chick Embryo Fibroblast</td>
</tr>
<tr>
<td>CMC</td>
<td>CarboxyMethyl Cellulose</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon Dioxide</td>
</tr>
<tr>
<td>CPE</td>
<td>Cytopathic Effect</td>
</tr>
<tr>
<td>cRNA</td>
<td>Complementary RNA</td>
</tr>
<tr>
<td>CRS</td>
<td>Congenital Rubella Syndrome</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal Fluid</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>F</td>
<td>Fusion Protein</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal Calf Serum</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration, USA</td>
</tr>
<tr>
<td>HA</td>
<td>Haemaglutinin Protein</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>HN</td>
<td>Haemagglutinin-neuraminidase protein</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory Bowel Disease</td>
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<td>I.C.</td>
<td>Intracerebral Inoculation route</td>
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<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IMS</td>
<td>Industrial Methylated Spirits</td>
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<tr>
<td>I.S.</td>
<td>Intraspinal Inoculation route</td>
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<tr>
<td>JL</td>
<td>Jeryl Lynn strain</td>
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<tr>
<td>L</td>
<td>Large Protein</td>
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<tr>
<td>L-3</td>
<td>Leningrad-3 strain</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>------------------------------------------------</td>
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<tr>
<td>L-Zagreb</td>
<td>Leningrad-Zagreb strain</td>
</tr>
<tr>
<td>M</td>
<td>Matrix Protein</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum Essential Medium</td>
</tr>
<tr>
<td>MBP</td>
<td>Myelin Basic Protein</td>
</tr>
<tr>
<td>MM</td>
<td>Measles and Mumps Vaccine</td>
</tr>
<tr>
<td>MMR</td>
<td>Measles, Mumps and Rubella Vaccine</td>
</tr>
<tr>
<td>MNVT</td>
<td>Monkey Neurovirulence Test</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of Infection</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MSD</td>
<td>Merck Sharpe and Dohme</td>
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<td>MuV</td>
<td>Mumps Virus</td>
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<tr>
<td>MV</td>
<td>Measles Virus</td>
</tr>
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<td>NA</td>
<td>Neuraminidase Protein</td>
</tr>
<tr>
<td>NDV</td>
<td>Newcastle Disease Virus</td>
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<tr>
<td>NP</td>
<td>Nucleocapsid Protein</td>
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<tr>
<td>OPV</td>
<td>Oral Poliovirus Vaccine</td>
</tr>
<tr>
<td>ORF</td>
<td>Open Reading Frame</td>
</tr>
<tr>
<td>P</td>
<td>Polymerase Protein</td>
</tr>
<tr>
<td>Pfu</td>
<td>Plaque Forming Units</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
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<td>RNAP</td>
<td>RNA Promoter</td>
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<tr>
<td>RNP</td>
<td>Ribonucleoprotein</td>
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<td>RNVT</td>
<td>Rat Neurovirulence Test</td>
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<td>RSV</td>
<td>Respiratory Syncytial Virus</td>
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<td>RT-PCR</td>
<td>Reverse Transcriptase PCR</td>
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<tr>
<td>SeV</td>
<td>Sendai Virus</td>
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<tr>
<td>SH</td>
<td>Small Hydrophobic Protein</td>
</tr>
<tr>
<td>SKB</td>
<td>SmithKline Beecham</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>SSPE</td>
<td>Subacute Sclerosing Panencephalitis</td>
</tr>
<tr>
<td>SV5</td>
<td>Simian Virus 5</td>
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<tr>
<td>TCID</td>
<td>Tissue Culture Infectious Dose</td>
</tr>
<tr>
<td>TgPVR</td>
<td>Transgenic Mice containing the Poliovirus Receptor</td>
</tr>
<tr>
<td>VSV</td>
<td>Vesicular Stomatitis virus</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
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<tr>
<td>YF</td>
<td>Yellow Fever Virus</td>
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1 Introduction

1.1 General Introduction

A popular misconception concerning the mumps virus (MuV) is that it is the causative agent of nothing more than a trivial childhood illness. This concept may have arisen due to the age and familiarity of the disease. The first known documentation of mumps disease was in the 5th Century B.C by Hippocrates, who described a mild epidemic illness characterised by a non-suppurative swelling near the ears; this is clinically referred to as parotitis. In 1935, Johnson and Goodpasture confirmed a virus as the aetiological agent of the mumps disease. Further experimentation, using Johnson’s neighbour’s children, allowed them to define the incubation period of the virus close to 18 days. Johnson and Goodpasture continued their research and successfully isolated and propagated the virus in embryonated hens eggs. MuV transmission is via droplet spread and it can infect the nasal mucosa or upper respiratory mucosal epithelium. This results in viremia and the virus disseminates to most organ systems, including the central nervous system (CNS) (Rubin et al 1998).

Complications arising from viral dissemination include the invasion of visceral organs; epididymo-orchitis, meningo-encephalitis, acute or chronic encephalitis, pancreatitis, oophoritis, mastitis, myocarditis and spontaneous abortion (WHO Review 1994). The most serious complications occur in the post-pubescent population.

The mumps disease and its associated complications declined after the introduction of an attenuated mumps virus vaccine. The vaccine is a result of serial passaging of a live mumps virus in embryonated hens eggs. The mumps vaccine can be administered in a variety of forms, as a monovalent mumps vaccine, a bivalent measles and mumps vaccine, MM, or a trivalent MMR vaccine, a combined vaccine incorporating live attenuated measles and rubella in conjunction with mumps. The rubella virus is known to cause congenital rubella syndrome (CRS), which can lead to foetal defects if the virus infects a pregnant woman. The measles virus (MV) can indirectly cause immune suppression although the virus infection is self-
limiting. For the ease of administrating the three attenuated vaccine viruses they are combined together to generate a single vaccine preparation. The MMR vaccine was introduced into the UK immunisation schedule in 1988 and has been used successfully ever since to help maintain a protective population immunity.

As effective as the vaccines have proven to be, they remain restricted to more affluent countries due to logistic and economic reasons.

1.2 The Mumps Virus

1.2.1 Classification

MuV is a member of the super-family Mononegavirales, which contains the single-stranded non-segmented negative sense RNA viruses. Single-stranded RNA viruses are classified as being either positive or negative sense. The encapsidated molecule of a positive sense RNA virus is infectious by itself and corresponds to functional messenger RNA (mRNA). Negative sense viruses are complementary to the mRNA produced from them and are strictly dependent on viral transcriptase enzymes packaged within the virions to initiate the infectious cycle (Baltimore et al 1971). Members of the super-family Mononegavirales are non-segmented and comprise four families Bornaviridae, Rhabdoviridae, Paramyxoviridae and Filoviridae (Pringle and Easton, 1997). In this superfamily, there are not only those human and animal pathogens that have been recognised since early studies in virology, but also newly emerging agents, such as Nipah virus and Hendra virus.

Originally, MuV was classified in the Myxoviridae family alongside the influenza virus. This was primarily due to its ability to agglutinate red blood cells. Currently MuV is classified in the Rubulavirus genus of the Paramyxoviridae family (Rima et al 1995).

The name paramyo is derived from the Greek para meaning ‘close to’ (in reference to the families close relationship) and myxa meaning mucus (in reference to the site of infection).
Table 1.1 shows the present classification of the *Mononegavirales* and lists representative species from each genus. This classification system is based on the morphological characteristics, genome organisation and sequence relationships of the viruses.

Table 1.1: Representative virus species in the super family *Mononegavirales*

<table>
<thead>
<tr>
<th>Family/SubFamily</th>
<th>Genus</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Paramyxoviridae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paramyxovirinae</td>
<td>Respirovirus</td>
<td>Sendai Virus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Human Parainfluenza 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Human Parainfluenza 3</td>
</tr>
<tr>
<td></td>
<td>Morbillivirus</td>
<td>Measles Virus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rinderpest Virus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Canine Distemper Virus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phocine Distemper Virus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hendra Virus</td>
</tr>
<tr>
<td></td>
<td>Rubulavirus</td>
<td>Newcastle Disease Virus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Simian Virus 5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mumps Virus</td>
</tr>
<tr>
<td>Pneumovirinae</td>
<td>Pneumovirus</td>
<td>Human Respiratory Syncytial Virus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bovine Respiratory Syncytial Virus</td>
</tr>
<tr>
<td><strong>Rhabdoviridae</strong></td>
<td>Vesiculovirus</td>
<td>Vesicular Stomatitis Virus</td>
</tr>
<tr>
<td></td>
<td>Lyssavirus</td>
<td>Rabies Virus</td>
</tr>
<tr>
<td></td>
<td>Ephemerovirus</td>
<td>Bovine ephemeral fever virus</td>
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<tr>
<td></td>
<td>Cytohabdovirus</td>
<td>Lettuce necrotic yellow virus</td>
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<tr>
<td></td>
<td>Nucleohabdovirus</td>
<td>Potato yellow dwarf virus</td>
</tr>
<tr>
<td><strong>Filoviridae</strong></td>
<td>Filovirus</td>
<td>Marburg Virus</td>
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<tr>
<td></td>
<td></td>
<td>Ebola Virus</td>
</tr>
<tr>
<td><strong>Bornaviridae</strong></td>
<td>Bornavirus</td>
<td>Borna Disease Virus</td>
</tr>
</tbody>
</table>
1.2.2 Biological Characteristics

Mumps is an enveloped virion of approximately 150nm in diameter and is pleomorphic in shape. The virion contains the non-segmented negative-sense RNA in association with three viral proteins, the nucleocapsid protein (NP), polymerase protein (P) and the large protein (L). This nucleocapsid complex is also found associated with the membrane bound matrix protein (M). There are two surface glycoproteins embedded in the lipid bilayer known as the haemagglutinin-neuraminidase (HN) glycoprotein and the fusion (F) glycoprotein. The small-hydrophobic (SH) protein which is possessed by a few rubulaviruses, including mumps and simian virus 5 (SV5), is thought to be membrane associated. Though its actual function is unknown, it has been postulated to be involved in apoptosis in SV5 (He et al, 2001). As with other enveloped RNA viruses, such as the human immunodeficiency virus (HIV) and MV, the budding virion envelopes itself in the host plasma membrane and buds in to the extracellular medium adopting the membrane as its own. Figure 1.1 is a structural representation of the mumps virus.

1.2.3 Genomic Structure

The average MuV genome is approximately 15,384 nucleotides in length and encodes information for seven genes in the following order:

\[3' - \text{NP} - \text{P/V} - \text{M} - \text{F} - \text{SH} - \text{HN} - \text{L} - 5']

A fifty-five nucleotide non-coding region located at the 3′ terminus is called the leader sequence and a twenty-four nucleotide non-coding region located at the 5′ terminus is called the trailer sequence. The length of the genes and the protein products produced by them are shown in table 1.2. Transcription of the P gene gives rise to more than one translatable mRNA species, due to overlapping reading frames (ORF). These allow the virus to maximise its production of different proteins whilst minimising its genome length. The precise function of the V protein is unknown at present.
Figure 1.1: The structural representation of MuV.

Showing six of the proteins of MuV including the RNA genome. The SH protein has not been localised within the MuV and its precise function is unknown.
Table 1.2: Length of mumps virus genes and number of deduced amino acids (Elango et al 1989).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Nucleotide Length</th>
<th>Amino Acid Length</th>
<th>Expected Molecular Weight (Kd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP</td>
<td>1,845</td>
<td>549</td>
<td>69</td>
</tr>
<tr>
<td>P</td>
<td>1,312</td>
<td>391</td>
<td>45</td>
</tr>
<tr>
<td>M</td>
<td>1,253</td>
<td>375</td>
<td>40</td>
</tr>
<tr>
<td>F</td>
<td>1,721</td>
<td>538</td>
<td>65</td>
</tr>
<tr>
<td>SH</td>
<td>310</td>
<td>57</td>
<td>6.7</td>
</tr>
<tr>
<td>HN</td>
<td>1,887</td>
<td>582</td>
<td>80</td>
</tr>
<tr>
<td>L</td>
<td>6,925</td>
<td>2261</td>
<td>180</td>
</tr>
</tbody>
</table>
1.3 Viral Proteins

1.3.1 The Nucleocapsid (NP) Protein.

The NP protein associates with the P and L proteins to form a nucleocapsid complex; a single-stranded, left-handed helical structure of about 1μm in length which interacts with the RNA to form a ribonucleoprotein (RNP). The RNP complex is a flexible structure coiled within the virus particle and its function is thought to be the protection of genomic RNA from degradation. The RNA and the L and P proteins associate with the NP protein during transcription and replication; the intracellular concentration of unassembled NP controls this association and therefore, transcription and replication processes are indigenously self regulated.

The gene sequence of the NP protein has been described for many paramyxoviruses (Morgan et al 1984, Rozenblat et al 1985, Ishida et al 1986 and Elango et al 1989). The results of these studies conclude that the NP protein consists of two domains corresponding to its N and C termini. The N-terminus is a globular domain with an overall positive charge that increases the NP protein’s affinity for RNA binding and subsequently, RNA protection. The N-terminus is highly conserved amongst paramyxoviruses within the genus members (Liston et al 1997). NP-NP protein binding is also possible via the N-terminus and is essential for RNP formation (Liston et al 1997).

The C-terminus is not as well conserved among paramyxoviruses as the N-terminal region, and contains the protein phosphorylation and antigenic sites (Hsu and Kingsbury 1982). The C-terminus has also been shown to be responsible for mediating the binding of the P protein to nucleocapsids (Ryan and Porter 1990). By cleaving and removing the C-terminus from the NP protein, Heggeness et al (1981) showed that the C-terminus is not essential for RNA binding or protection.

1.3.2 The Polymerase (P) Protein.

The highly phosphorylated P protein is located within cytoplasmic inclusions. Its main function is to associate with the L protein to form the viral polymerase P-L and direct RNA
synthesis in the cytoplasm. The P-L polymerase complex also associates with unassembled NP protein to form the active complex of RNA encapsidation (Horikami et al 1992). The length of the P gene has been shown to be variable amongst paramyxoviruses and also within the rubulavirus genus, its size ranging from 245-397 amino acid residues. Transcription gives rise to more than one mRNA species from the genomic region corresponding to the designated P gene. The V protein is translated from an mRNA in which the ORF (for the P region) is altered by editing. Studies of a V protein plasmid construct by Kato et al (1997) showed that the V protein is not essential for replication. The V protein in SV5, a close relative to mumps virus, has been shown to inhibit interferon (IFN) signalling (Didcock et al 1999). The authors concluded that IFN inhibition allows the virus to replicate in vivo. The N-terminal region of the P protein contains protein phosphorylation sites (Vidal et al 1988) and regions essential for RNA synthesis, including a unique region for RNA encapsidation (Curran et al 1994). RNA editing in the morbillivirus genera also gives rise to another protein, designated the C protein. The C protein is a small protein which is located in the nucleus. Its function is unknown although both V and C proteins are expressed in infected cells (Bellini et al 1985).

1.3.3 The Matrix (M) Protein.

The M protein is membrane associated and plays a role in virion assembly. The nucleotide sequence for the M protein has been established for many paramyxoviruses (Blumberg et al 1984, Chambers et al 1986, Afzal et al 1994). The M protein is synthesised by free ribosomes and is located beneath the viral lipid bilayer. It is a basic protein containing hydrophobic domains that are too small to span the lipid bilayer, thus concluding that the M protein is associated peripherally with the membrane. The primary function of the M protein is thought to be structural, aiding the organisation of the virion prior to budding from the infected cell (Yoshida et al 1976, Matsumoto 1982). The MuV M protein associates with the cytoplasmic tails of the envelope glycoproteins, the lipid bilayer and the nucleocapsid. The M protein can also self-associate, a characteristic which is
thought to be essential in budding virion formation. Mottet et al (1996) observed that deletions in the SeV M protein hydrophobic region, resulting in an overall negative charge, prevented M protein self-association. Different deletions to give the M protein an overall positive charge increased self-association. In the persistent infection sub-acute sclerosing panencephalitis (SSPE) the MV M protein is produced at normal rates; however, it has been shown to be very unstable and dissociates rapidly. This rapid dissociation means that the virus is unable to bud and therefore the infection remains persistent (Hirano et al 1992). It has been shown that the M protein of wild-type MV can bind to the nucleocapsids of SSPE strains, concluding that M protein of SSPE viruses is defective in nucleocapsid-binding (Hirano et al 1993).

1.3.4 The Fusion (F) Protein.

As its name suggests, the F protein is involved in virus-to-cell and cell-to-cell fusion (Bratt and Gallaher et al 1969). It is one of the proteins responsible for the formation of large multi-nucleated cells, called syncytia, which are characteristic of mumps infection. The F protein is a classic type I integral membrane protein, which is classified as having its N-terminus external to the virion and which spans the membrane once. Its structure consists of three domains; a large hydrophilic domain which is external to the virion, a domain of twenty uncharged amino acid residues which serves to anchor the protein to the membrane and a second hydrophilic domain located on the inside of the virion membrane.

The F protein is synthesised as a precursor F₀. This precursor is cleaved by a host cell protease, called Furin, into two biologically active units F₁ and F₂ which are linked by a disulphide bond (Scheid and Choppin 1977). Cleavage of F₀ is essential for infectivity and pathogenicity.

The N-terminus of paramyxovirus F₁ proteins has twenty hydrophobic conserved amino acid residues, termed the fusion peptide. The fusion peptide is usually hidden as part of an α-helix, except during fusion where conformational changes occur allowing exposure of the peptide to interact with the cell membrane (Chambers et al 1990). The same is also true for
other viral fusion proteins, such as the influenza haemaglutinin (HA) protein (Chan et al 1997), the HIV gp41 fusion protein (Joshi et al 1998) and the Ebola gp2 fusion protein (Russell et al 1994). There are many similarities between these fusion peptides; they are all homotrimers (Russell et al 1994) that require a conformational change to occur in order to become active. The F protein is glycosylated (Lambert 1988) and in MuV there are seven possible sites for N-linked glycosylation but to date there is no evidence to suggest which of these sites is used.

In order for syncytium formation to occur there has to be expression and interaction between the F protein and the HN protein (Tanabayashi et al 1992); this has also been observed for Newcastle disease virus (NDV) (Morrison et al 1991).

It is unknown how the fusion protein interacts with the cell membrane to cause cell fusion.

1.3.5 The Small Hydrophobic (SH) Protein.

The SH protein is present in MuV and SV5. It is a hydrophobic, integral membrane protein which is located in the plasma membrane (Takeuchi et al 1996). The function of the SH protein is unknown; deletion of the gene from an infectious cDNA clone of SV5 found that the SV5 virus could be successfully recovered. The authors concluded that the SH protein is not necessary for virus replication in tissue culture (He et al 1998).

Sequence analysis has shown that the SH gene is highly variable between strains of MuV and is a useful region of the genome for molecular epidemiological investigations. Analysis of sequence data generated for many MuV strains (Afzal et al 1997, Orvell et al 1997) suggests that the MuV isolates can be grouped into six genotypes A to F (Wu et al 1998, Tecle et al 1998). The data show that sequences within the ORF are highly variable but that there is a highly conserved region of approximately 50 nucleotides preceding the ORF translation initiation site.
1.3.6 The Haemagglutinin-Neuraminidase (HN) Protein

The HN glycoprotein is a type II protein whose N terminus is located internally and spans the membrane once. Its function involves binding the virus to the host cell via sialic acid containing molecules on the cell surface (Markwell and Paulson 1980). This binding is strong and gives the virus the ability to agglutinate red blood cells of certain mammalian and avian species. The neuraminidase feature of the protein prevents self-aggregation of the virus after budding and also prevents re-adsorption back onto the infected cell. The HN protein is similar to the influenza neuraminidase (NA) protein, which is also a type II protein (Varghese et al 1983). The MuV HN protein is an oligomer consisting of disulphide bonds and forming tetramers. The site(s) for receptor binding is unidentified at present. It is suggested that HN protein binding to a receptor initiates a conformational change in the associated F protein releasing the hydrophobic fusion peptide into the target membrane (Stone-Hulslander and Morrison 1997).

The HN protein is a major antigenic determinant. It is the protein against which neutralising antibodies are raised. Studies using monoclonal antibody resistant (MAR) mutants have been conducted to determine the antigenic sites on the HN protein (Yates et al 1996).

1.3.7 The Large (L) Protein.

The L protein is the largest and least abundant of the viral proteins. The main role of the L protein is to play a part in the RNA-dependent RNA polymerase activity essential for viral transcription and replication. It is localised in the transcriptionally active viral core.

Nucleotide and amino acid sequences have been determined for many of the L protein genes of paramyxoviruses (Kawano et al 1991, Higuchi et al 1992). They are of similar length, approximately 2,200 amino acid residues, and there is little homology outside of the subfamilies. Towards the centre of the gene were found five regions of homology that are also conserved in other RNA-dependent RNA polymerases from other virus families (Poch et al 1990). These regions are assumed to be crucial for the enzymatic function of the protein. The P and L proteins combine to form a P-L complex which is required for polymerase
activity with the NP-RNA template (Hamaguchi et al 1988). A transcriptionally active nucleocapsid contains 5 to 10 P protein residues per one L protein residue. The L protein is also thought to be responsible for the addition of the polyA tail of the mRNA and the methyl-G capping of post-transcriptional mRNAs (Chinchar and Portner 1981).

1.4 Replication

For initiation of the infectious cycle, the virus must first enter a target cell. This is achieved by the virus surface glycoproteins attaching to the host cell receptor. The virus receptors are sialic acid containing macromolecules which occur on the host cell surface and specifically bind the virus (Markwell et al 1984). These macromolecules are common to most cells making viral entry possible to a variety of cell types. Two paramyxoviruses that do not use sialic acid containing molecules as receptors are respiratory syncytial virus (RSV) which binds to heparin sulphate (Feldmann et al 2000) and MV which uses a specific protein as a receptor (Naniche et al 1992).

After attachment, the viral membrane and host cell plasma membrane fuse at a neutral pH. This occurs through the interaction of the viral F protein with the host cell plasma membrane. The viral nucleocapsids are released into the host cell cytoplasm. The F protein must undergo a conformational change to become active. It is thought that, for MuV, this change occurs through interaction with the HN protein. The events of viral entry have been studied more extensively for the case of influenza virus (Klenk et al 1975). After attachment to the host cell via the sialic acid receptors, the influenza virus is internalised by receptor mediated endocytosis. After endocytosis has occurred, the influenza virus is transported to the lysosomes where an acidic pH environment causes an irreversible conformational change in the HA protein. This viral entry pathway is of interest for antiviral drug design.

The next stage in the cycle is transcription of the negative sense genomic RNA into mRNA. Paramyxoviruses use a single transcriptional unit with one promoter sequence. In Rubulaviruses, the 3’ end of the genome and the first gene, the NP gene, are separated by 55 nucleotides, the leader sequence. The first twelve nucleotides of the leader sequence are
conserved throughout the Paramyxoviruses. The viral P-L polymerase initiates the transcriptional process at the 3' end of the RNA and, after transcribing the leader sequence, the viral polymerase terminates and reinitiates transcription at the beginning of the first gene. Reinitiation of transcription after each termination sequence is not 100%, leading to a higher production of mRNA's located near to the leader sequence in comparison to L mRNA, the farthest from the leader sequence. Bicistronic and polycistronic mRNA's are also produced. By doing this the virus can control the relative levels of mRNA production.

The switch from transcription to replication is not well understood. Pattnaik et al (1995) showed that the signals needed for RNA replication are contained within the 36 nucleotides at the 5' genomic end of VSV. Whelan and Wertz (1999) concluded that elements at the 3' end are essential for transcription, but not replication. Abolishing the leader sequence promoted replication concluding that the leader sequence is less efficient as a promoter for replication than the trailer sequence.

Replication of the negative sense genome requires a full-length positive sense intermediate cRNA generated by the viral polymerase which copies the RNP template ignoring transcription stop signals. Kolakofsky and Ortin (1991) suggested this override could be caused by the levels of NP and RNA synthesis occurring concomitantly. With low levels of unassembled NP present, the polymerase preferentially engages in mRNA synthesis to raise the levels of all virus proteins. If levels of unassembled NP are high, the polymerase preferentially engages in genome replication. Genomic RNA synthesis is thought to occur in the same way as antigenome synthesis.

Nucleocapsids assemble in the cell cytoplasm resulting in the production of the helical RNP complex (Kolakofsky et al 1998). After RNP assembly, interaction with the P-L complex occurs. Proteins are transported through the ER and Golgi apparatus. Once all the proteins have been post-translationally modified, they assemble together near the surface of the host cell. This is achieved in paramyxoviruses by altering the chemical composition of an area of the plasma membrane making it more rigid. Viral glycoproteins are inserted into this area of
the membrane. The matrix protein accumulates on the inside of the membrane at this point and mediates interaction with the RNP. The whole complex then buds away from the plasma membrane into the intracellular environment. (Harper 1994)

1.5 Reverse Genetics of Paramyxoviruses

Viruses and their interactions with host cells are of great interest to the virology community. Within the past decade scientists studying mumps and other negative strand viruses and these interactions have had the benefit of a technique called Reverse Genetics – the ability to recover infectious virus from cloned DNA.

Genomic manipulations of viruses which use DNA as their genetic material were the first to be conducted in order to ‘rescue’ a virus in vitro. This recovery can be achieved either by transfecting cells with plasmids encoding the viral genome or by heterologous recombination of plasmids bearing sequences of the virus genome.

Rescuing infectious positive strand RNA viruses such as poliovirus, requires either the introduction into cells of in vitro synthesised RNA from cDNA or intracellular expression of RNA following DNA transfection. The plasmid-based reverse genetics technology was established for DNA and positive strand RNA viruses with relative ease.

The genomes of the negative strand RNA viruses have been less amenable to artificial manipulation for several reasons: (i) the viral RNA itself is non-infectious; (ii) precise 5’ and 3’ ends are required for replication and packaging of the genomic RNA: (iii) the viral RNA polymerase is essential for transcribing both mRNA and the cRNA intermediate; and (iv) both genomic and antigenomic RNA's exist as RNP complexes, which are crucial to the initiation of the viral life cycle. Virus rescue from cDNA for Mononegavirales has required years of development, much of which is still ongoing.
Figure 1.2: Diagrammatic representation of Paramyxovirus replication.

Detailing the two courses MuV RNA takes after penetration of the host cell membrane, all of which takes place within the cytoplasm of the infected cell.
The phenotypes of rescued viruses can be studied and defined in the context of not only viral replication in vitro but also in vivo viral multiplication and pathogenesis. With the aid of reverse genetics, researchers can address pending scientific questions which could not be addressed through conventional virology, for example in determining the function of the paramyxovirus accessory genes, C and V. Reverse genetics has opened many possibilities in the field of viral vaccine research; the designing of live viral vaccines with predetermined or plausible attenuating mutations and reducing the time needed to generate new vaccine viruses to a few weeks rather than the months or years that are required using conventional approaches. The technology required to produce an infectious clone of the mumps virus is now well established (Clarke et al 2000) and can be used in structure related functional studies as well as designer vaccines.

1.6 Clinical Symptoms of Mumps Infection

Mumps is an acute infectious disease which spreads through respiratory droplets. The disease can vary from a mild upper respiratory illness to viraemia with widespread systemic involvement (Table 1.3). Classic mumps is characterised by the enlargement of the parotid and other salivary glands (Baum and Litman 1995), although infection is asymptomatic in about one third of cases. Up to 10% of mumps patients develop aseptic meningitis. A less common but more serious complication is encephalitis, which can result in death or permanent disability. Deafness, orchitis and pancreatitis are other untoward effects, all of which are preventable through vaccination (Galazka et al 1999).

The clinical consequences of systemic infection can be quite severe if the illness is delayed until adulthood. Twenty-five percent of adult men who contract mumps suffer from epididymo-orchitis, the median age for which is 29 according to a large cohort study conducted in 1977 (Beard et al 1977). A further one-third of patients suffering from mumps orchitis also develop testicular atrophy, though complete sterility is rare. A possible link between mumps orchitis and testicular cancer has been demonstrated, though it is not considered to be a major cause but a risk factor (Swerdlow et al 1987). In
adult women, mumps mastitis and oophoritis can occur. A study by Philip et al (1959) showed that mumps mastitis occurs in 31% of women over the age of 14. Twenty-five percent of women who acquire MuV during the first 12 weeks of pregnancy suffered from spontaneous abortion, a rate which supercedes that due to rubella infection (Siegel et al 1966). Pancreatitis occurs in 4% of clinical mumps patients, with evidence to suggest that MuV infection of the human pancreatic beta cells can trigger the onset of insulin-dependent diabetes mellitus in some individuals (Stratton et al 1994).
Table 1.3: Clinical manifestations of mumps (modified from Baum and Litman 1995).

<table>
<thead>
<tr>
<th>Manifestation</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Glandular</strong></td>
<td></td>
</tr>
<tr>
<td>Parotitis</td>
<td>60-70</td>
</tr>
<tr>
<td>Submandibular and/or sublingual adenitis</td>
<td>10</td>
</tr>
<tr>
<td>Epididymo-orchitis</td>
<td>25 (Post-pubertal men)</td>
</tr>
<tr>
<td>Oophoritis</td>
<td>5 (Post-pubertal women)</td>
</tr>
<tr>
<td>Pancreatitis</td>
<td>4</td>
</tr>
<tr>
<td><strong>Neurological</strong></td>
<td></td>
</tr>
<tr>
<td>Aseptic meningitis</td>
<td>1-10</td>
</tr>
<tr>
<td>Encephalitis</td>
<td>0.02-0.3</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td></td>
</tr>
<tr>
<td>Mild Renal function abnormalities</td>
<td>30-60</td>
</tr>
<tr>
<td>Electrocardiagram abnormalities</td>
<td>5-15</td>
</tr>
<tr>
<td>Deafness (usually transient)</td>
<td>4</td>
</tr>
</tbody>
</table>
Using saliva from an infected patient, meningoencephalitis was reproduced in macaques inoculated by the intracerebral (i.c) route as far back as in 1934 (Gordon 1934), and before the widespread use of a mumps vaccine, MuV was among the most common causes of hospitalisation from meningitis (Russell and Donald 1958).

Up to 10% of mumps patients develop aseptic meningitis and, for unknown reasons, more often in males than females. Mumps meningitis is clinically manifested by a severe headache that is aggravated by movement, photophobia and neck stiffness due to spasms of the spinal muscles (Grist 1993). This is a benign condition that appears within a few days of parotid swelling; however, not all meningitis patients exhibit parotitis. Mumps meningitis patients generally recover without complications, though many require hospitalisation for the duration of the illness. In Sweden during the pre-vaccine era, it was estimated that annually, mumps caused approximately 1000 meningitis cases. This in turn led to 20,000 days of hospitalisation and 20,000-40,000 days of disability (Bjorvaten and Skoldenberg 1979). The incidence of mumps encephalitis is reported to range from 1 in 6000 mumps cases (Russell and Donald 1958) to 1 in 300 mumps cases (CDC 1974). Encephalitis symptoms range from mild alterations of consciousness to coma, emotional liability and irritability. For as yet unknown reasons, mumps encephalitis affects three times as many men as women (CDC 1974).

A well-recognised complication of mumps infection is deafness. In Finland, 298 military personnel who contracted MuV were assessed for deafness using audiometric tests. The results showed that 13 (4%) had high frequency hearing loss. The hearing loss reverted within a few weeks for 12 but one progressed to permanent deafness (Vuori et al 1962). Further clinical symptoms observed with MuV infection include mild renal function abnormalities (Utz et al 1964) that generally resolve spontaneously. Transient electrocardiogram abnormalities occur in 15% of cases (Rosenberg 1945) with a rare case of fatal myocarditis also having been reported (Ozkutlu et al 1989).

Fatality attributed to MuV is exceedingly rare and when it occurs is mainly due to mumps...
encephalitis. In the USA, from 1966-71, there were two deaths per 10,000 mumps cases with 38% of such deaths involving persons aged >40 years (CDC 1974). In the UK, 93 deaths were registered from mumps over the period 1962-81 with 53 (57%) of those who died being aged >45 years (Galbraith et al 1984).

1.7 Mumps Vaccines History

All mumps vaccines consist of a live attenuated strain of virus. Live attenuated mumps vaccines are available as monovalent mumps vaccine, bivalent measles-mumps, MM, vaccine and trivalent measles-mumps-rubella, MMR, vaccine. World Health Organisation (WHO) requirements do not specify the minimum amount of mumps vaccine virus that one human dose should contain; rather, this is determined by the national control authority of the country where the vaccine is licensed (WHO Tech Rep 1994). The majority of countries use 1000 Tissue Culture Infectious Doses 50% (TCID\textsubscript{50} – meaning the dose required for 50% of tissue culture cells to be infected with the virus) of attenuated mumps virus per dose. Live mumps vaccines are produced as lyophilised powder and once they have been reconstituted with sterile water they must be used immediately or stored at 0-8°C away from light and discarded within 8 hours (WHO Tech Rep 1994).

Since its introduction, several strains of MuV have been used in the mumps vaccine. In the UK, the MMR vaccine currently contains the attenuated Jeryl Lynn (JL) strain, named after the child from whom it was isolated in 1965. The virus was attenuated by passaging in embryonated hens eggs and subsequently in chick embryo cell culture (Buynak and Hilleman 1966). The strain was licensed in 1967 and by 1992 it had been administered to over 135 million children and adults worldwide (WHO Tech Rep 1994). Clinical studies undertaken in industrialised countries have shown that one dose of the JL strain of mumps vaccine leads to an initial seroconversion rate of 80-100%, which increases to 97-100% with two doses (Fahlgren 1988). Further studies documented antibody persistence in a large proportion of vaccinees. A Swedish study found that 73% of children who received two doses of the MMR vaccine containing the JL strain at 18 months of age remained seropositive 10.5 years later.
The protective efficacy of 1 dose of the JL strain in vaccine outbreak-based studies has ranged from 75-91% (Cochi et al 1994). There are few figures for JL vaccine efficacy in developing countries; however, a study in the Dominican Republic reported a 94% seroconversion rate among 72 seronegative children aged 1-6 years old (Ehrenkranz et al 1975).

In studies of vaccine associated adverse events in the United States of America (USA), a 10 year study of hospitalised cases in a cohort of children due to MuV infection found one case of aseptic meningitis per 100,000 doses of JL containing MMR vaccine (Black et al 1997). In Germany, the JL strain was associated with 0.1 aseptic meningitis cases per 100,000 vaccine doses (Fescharek et al 1996). These reports conclude that there is a risk of developing aseptic meningitis from the mumps component of the MMR vaccine however the incidence is very low. Many other strains of attenuated MuV have also been used in vaccines. The live attenuated Urabe strain of MuV was first licensed in Japan in 1979 and thereafter in Belgium, France, UK, Canada and Italy (WHO Tech Rep 1994). It was attenuated by serial passaging in the amnion of embryonated hen's eggs. By 1991, more than 60 million people worldwide had been immunised with this vaccine (WHO Tech Rep 1994).

A Finnish study of children who received a mumps vaccine at 14-20 months of age showed 95% seroconversion with the Urabe vaccine as compared to 97% with the JL vaccine (Vesikari et al 1983). Studies that assessed the immunogenicity of the Urabe vaccine among seronegative children in developing countries reported 99% seroconversion in Brazil (Forleo-Neto et al 1997) and 98% in South Africa (Schoub et al 1990). A Canadian study reported that 5-6 years after one dose of MMR vaccine the seropositivity rate was 93% for the Urabe vaccine, compared with 85% for the JL vaccine (Boulianne et al 1995).

The fears that some children developed aseptic meningitis after receiving the Urabe containing MMR vaccine initiated a study using molecular tools to investigate any possible relationship between the vaccine and associated adverse events. Spinal fluid analysed from vaccinees who developed aseptic meningitis confirmed the association of the Urabe strain to
the disease (Brown et al 1996). This led to Urabe containing MMR vaccines being withdrawn from the market in Canada by 1990 (Furesz and Contreras 1990).

Following this report, the UK conducted studies into the rates of aseptic meningitis that were attributable to the use of the Urabe vaccine. A small study in Nottingham was followed by a multi-centre confirmatory study that confirmed 9 cases of aseptic meningitis per 100,000 vaccine doses (Miller et al 1993), as a result of which the UK Public Health Service Department stopped purchasing the Urabe vaccine in 1992.

The Rubini strain of mumps vaccine was attenuated by passaging in MRC-5 human diploid cells followed by serial passaging in embryonated hen's eggs and then adaptation back to the MRC-5 cell line (Glück et al 1986). In 1985, Switzerland licensed a mumps vaccine based on the Rubini strain and by 1990 it had been used to vaccinate over 4 million people worldwide (WHO Tech Rep 1994). A study conducted in Switzerland provided evidence that the Rubini mumps vaccine did not offer long-term protective immunity against the disease. The study found an efficacy rate of 6% over 16 years, compared to 62% for the JL strain and 73% for the Urabe strain (Chamot et al 1998).

The attenuated Leningrad-3 (L-3) vaccine was developed in the former Soviet Union in guinea-pig kidney cell cultures (Smorodintsev et al 1970) and subsequently licensed in 1974 in the USSR. Approximately 8-11 million doses of the L-3 vaccine were produced annually (WHO Tech Rep 1994). Studies on seroconversion and the efficacy of the L-3 vaccine showed a 89-98% seroconversion rate and a protective efficacy of 92-99% (Smorodintsev et al 1970). In Slovenia, passive surveillance from 1979-1985 identified 20 cases of aseptic meningitis per 100,000 doses of MM vaccine containing the L-3 strain (Kraigher 1990). The Leningrad-Zagreb (L-Zagreb) strain was produced in Croatia by further adapting the L-3 MuV through passage on CEF cells (Beck et al 1989). Over the period of 1975-87, more than 10 million doses of L-Zagreb mumps vaccine were distributed in the former Yugoslavia (Beck et al 1989). Studies in Croatia conducted on L-Zagreb vaccine showed 87-100% seroconversion and an efficacy of 97-100% (Beck et al 1989). However, there were 90 cases
of aseptic meningitis per 100,000 doses of L-Zagreb containing MMR vaccine over the period of 1988-92. In Slovenia, MMR vaccines containing the L-Zagreb mumps strain have been used since 1990. Passive surveillance over the period 1990-96 revealed two cases of aseptic meningitis per 100,000 doses (Kraigher 1990).

Mumps was a common infectious disease in the pre-vaccine era, with a high annual incidence >100 per 100,000 population based on routine passive surveillance (Table 1.4) whilst incidences greater than 6000 cases per 100,000 have been reported in military populations (Gordon and Kilham 1949). Countries which lack a mumps vaccination program display a high incidence of the disease, with epidemic peaks occur every 2-5 years with the infected majority being those aged 5-9 years.
Table 1.4: Average annual reported mumps incidence in the WHO European Region before and after the introduction of mumps vaccine including two countries with no mumps vaccination (Adapted from Bakasun 1997).

<table>
<thead>
<tr>
<th>Country</th>
<th>Pre-vaccine</th>
<th>Post- vaccine</th>
<th>% Reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Years</td>
<td>Average annual Incidence *</td>
<td>Years</td>
</tr>
<tr>
<td><strong>Two-Dose Schedule</strong></td>
<td></td>
<td></td>
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<tr>
<td>Denmark</td>
<td>1977-79</td>
<td>726</td>
<td>1993-95</td>
</tr>
<tr>
<td>Finland</td>
<td>1977-79</td>
<td>223</td>
<td>1993-95</td>
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<tr>
<td>Norway</td>
<td>1977-79</td>
<td>371</td>
<td>1993-95</td>
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<tr>
<td>Slovenia</td>
<td>1977-79</td>
<td>410</td>
<td>1993-95</td>
</tr>
<tr>
<td>Sweden</td>
<td>1977-79</td>
<td>435</td>
<td>1993-95</td>
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<tr>
<td><strong>One-Dose Schedule</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Armenia</td>
<td>1983-85</td>
<td>280</td>
<td>1993-95</td>
</tr>
<tr>
<td>Croatia</td>
<td>1983-85</td>
<td>101</td>
<td>1993-95</td>
</tr>
<tr>
<td>England and Wales</td>
<td>1983-85</td>
<td>40</td>
<td>1993-95</td>
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<tr>
<td>Israel</td>
<td>1983-85</td>
<td>102</td>
<td>1993-95</td>
</tr>
<tr>
<td>Latvia</td>
<td>1983-85</td>
<td>141</td>
<td>1993-95</td>
</tr>
<tr>
<td><strong>No Mumps Vaccine</strong></td>
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<tr>
<td>Poland</td>
<td>1983-85</td>
<td>415</td>
<td>1993-95</td>
</tr>
<tr>
<td>Romania</td>
<td>1983-85</td>
<td>242</td>
<td>1993-95</td>
</tr>
</tbody>
</table>

* cases per 100,000 population
Prior to the introduction of mumps vaccine, serosurveys conducted to assess population immunity to MuV showed that protective maternal antibody is passively transferred to the infant, but only has a half-life of 35-40 days. Data collected from England and Wales (Morgan-Capner et al 1988), the Netherlands (Wagenvoort et al 1980) and Singapore (MMR Report 1992) document an increase in mumps antibody level from the age of 2-3 years; by 4-6 years of age 50% of children had acquired natural antibodies and by 14-15 years of age, 90% of the population was seropositive. It is not recommended to administer mumps vaccines to immunosuppressed individuals or to pregnant women due to the theoretical risk of foetal damage, and pregnancy should be avoided for a minimum of three months post-vaccination (Morb and Mort Rep 1998).

In recent years, there has been much speculation as to the relative safety of MMR vaccine. A report published by Wakefield et al in 1998 raised concerns of a possible link between the persistence of MV in the gut and inflammatory bowel disease (IBD). Since then, many scientific teams have researched this claim. Teams using highly sensitive and specific reverse transcription polymerase chain reaction (RT-PCR) methods to examine colonic biopsies from newly diagnosed and treated Crohn’s disease patients and resection specimens, targeting various regions of the MV genome, N, F, M and H genes, found no evidence of MV in the gut (Haga et al 1996, Afzal et al 1998a, 1998b, 2000, Chadwick et al 1998). There are reports in support of the Wakefield et al claim (Kawashima et al 2000). However, a lot of the results are inconsistent and there is overwhelming research and epidemiological evidence to the contrary (Davis et al 2001).

Wakefield et al (1998) also suggested a relationship between MMR vaccine and autism. Autism is a syndrome of unknown aetiology and many factors have been considered to be potential risks, including environmental influences, genetic predisposition, viral infection and immunological reactions (Deykin 1979, Singh 1994, Folstein and Rutter 1988). Evidence for the presence of auto-antibodies to myelin basic protein (MBP) in autistic individuals has been shown (Singh et al 1993). These authors also showed that 90% of
measles IgG positive autistic sera contain anti-MBP antibodies, suggesting a link between vaccination and autism. Chicken MBP residue could be present in MMR vaccine preparations as the vaccine is propagated in cells derived from eggs and antibodies against chicken MBP can cross react with human MBP. However, Afzal et al (2001) found a lack of chicken MBP residues in any commercial formulations of MMR vaccine. Signs of the autism syndrome appear around 15-20 months of age, which coincides with the recommended age of MMR vaccination (MMWR, 2000). Although there has been overwhelming scientific evidence showing the lack of a possible relationship between MMR vaccination, IBD and juvenile autism, the faith of the public on MMR vaccine safety remains low causing a decline in vaccine uptake at a national level. It is possible that if the vaccine uptake does not improve significantly then the UK may encounter an epidemic of measles, mumps and rubella.

1.8 Neurovirulence of Mumps Virus and Mumps Vaccines

Mumps virus is a neurotropic virus of humans (Zuker 1975) with up to 10% of mumps infected patients developing meningitis, and encephalitis due to MuV occuring in up to 0.3% of infected patients. Mumps meningitis involves the meninges and can often take an asymptomatic course (Ocklitz 1976). The involvement of the central nervous system during mumps infection has been one of the major reasons for developing preventative immunisation against this infection. Consequently the neurotropic nature of the vaccine virus must be considered when assessing the safety of live mumps vaccines and a reliable neurovirulence test is, therefore, desirable. Such testing is a requirement as stipulated by the European Pharmacopoeia and health organisations of countries with mumps vaccination programmes. Experiments conducted on hamsters (foetal, newborn and adult) showed that pathogenic wild-type and vaccine derived MuV has a high tropism towards the ventricular ependyma with the possibility of hydrocephalus development (Johnson et al 1967, Johnson and Johnson 1969, 1972, Kilham and Margolis 1975). An aetiological role of MuV in hydrocephalus in man is also suspected (Timmons and Johnson 1970, Bray 1972, Johnson and Johnson 1972). In 1979, Levenbruk et al performed experiments on monkeys using
variants of attenuated MuV. These viruses retained a more or less substantial degree of virulence which was indicated by the specific morphological lesions localised away from the inoculation site. At a higher attenuated passage level, they obtained a variant causing much more pronounced changes in the brain ventricular system. This variant was regarded as the variant with the greatest risks, from the viewpoint of its higher tropism towards the ventricular structures.

1.9 Structure of the Brain

The vertebrate brain is divided into three regions; the forebrain, the midbrain and the hindbrain. Each of these regions receives and analyses sensory information from a particular type of sensory receptor. For example, the forebrain receives chemical information from the nose via the olfactory nerves, the midbrain receives visual information from the eyes via the optic nerve, and the hindbrain receives vibration and orientation information from the ears and balance organs via the auditory and vestibular nerves, respectively. Each of these regions contains groups of neuronal cell bodies which are collected together to serve a particular function. The central core of the midbrain and the hindbrain is called the brain stem.

1.9.1 The Ventricles

The ventricles are the remnants of the tube of hollow fibres from which the brain developed. They lie deep inside the forebrain, midbrain and hindbrain in a series of inter-connecting chambers lined with an epithelium called ependyma. These ventricles are filled with a liquid called the cerebrospinal fluid (CSF), which also surrounds the brain, functioning to cushion it from knocks. CSF is constantly produced and percolates through and fills the ventricles. It leaves the system by flowing out of three openings from the fourth ventricle. The CSF does not contain red blood cells and its composition is different from the composition of the fluid part of the blood, plasma. This difference is the result of the activity of special cells which form what is known as the blood-brain-barrier. The existence of the blood-brain barrier
protects the neurons in the CNS from harmful substances; it also affects how easily drugs can enter the extracellular environment of the CNS.

1.10 Neurovirulence Testing of Live Viral Vaccines

Live viral vaccines must undergo neurovirulence testing before being released onto the general market. One of the objectives in regulating medicinal products is to control the safety and efficacy of material being administered. This is essential in order to document any adverse events prior to distribution.

1.10.1 Yellow Fever (YF) Vaccine

Neurological complications arising from the 17D strain of yellow fever vaccine have been well documented (Fox and Penna 1943), and a monkey safety test is used to assess the neurovirulence of YF vaccine virus. This test is based on the clinical manifestations of encephalitis observed in i.c inoculated monkeys (WHO Tech Rep 1976). Various seed viruses prepared from different passage levels of this strain displayed different degrees of neurovirulence in monkeys when inoculated into the CNS. One human dose of the vaccine (5000-50,000 Mouse Lethal Doses$_{50}$) is inoculated into the frontal lobe of the monkey brain. The monkeys are observed for a period of 30 days for signs of encephalitis. According to WHO requirements (1976) the vaccine has an acceptable degree of neurovirulence if not more than 10% of inoculated monkeys develop severe signs of encephalitis; these include paralysis, inability to stand when stimulated or death. In addition, the test virus must not induce other signs of encephalitis, such as incomplete paralysis, incoordination, tremors, lethargy, inability to stand spontaneously, motor weakness or spasticity, at a frequency greater than that induced by a laboratory reference vaccine with acceptable properties in man and monkey. A grading method is used in order to assign a numerical value to the severity of the clinical signs exhibited. The accepted current grading method was developed by Levenbook et al (1987) and is based on observations of monkeys inoculated with YF vaccine virus:
Grade 1: rough coat, not eating

Grade 2: high pitched voice, apathetic, inactive, slow moving, general weakness.

Grade 3: shaky, tremors, uncoordination, limb weakness.

Grade 4: inability to stand, upper or lower limb paralysis, or death.

1.10.2 Live Polio Vaccine

The oral polio vaccine (OPV) has a well documented viral neurovirulence test and the vaccine is used throughout the world in the WHO initiative to eradicate the disease, poliomyelitis. A high degree of attenuation is a basic requirement for OPV. Assessing the degree of attenuation of the three subtypes of poliovirus (1, 2 and 3) is achieved by inoculation of a test virus into the CNS of monkeys. The monkey neurovirulence test (MNVT) has played a crucial role in the development and maintenance of the live vaccine and the MNVT has proven to be a sensitive test which assures the safety of OPV in humans (Contreras et al 1988). In 1982, a new standardized MNVT for the control of OPV was developed and adopted by the WHO (WHO Tech Rep 1990). An MNVT on each monovalent bulk of OPV is a mandatory requirement of the WHO and the national authorities. This differs from mumps vaccines where it is only the seed stock that requires neurovirulence testing and not each bulk. However, the use of the large numbers of monkeys required in each MNVT poses increasing ethical problems, health risks to animal keepers and high costs. In light of these disadvantages it would be desirable to replace monkeys with an \textit{in vitro} test.

In 1989-1990, two groups of scientists (Ren et al 1990, Koike et al 1991) with the support of the WHO Vaccine Development Program, genetically engineered mice to make them susceptible to poliovirus. These mice were designated TgPVR mice – transgenic for the poliovirus receptor. Both groups recommended these animals to replace the monkey model for investigation of poliovirus neurovirulence attenuation. In 1991, the WHO recommended that a study be conducted in order to compare the suitability of replacing monkeys with TgPVR mice and the USA Food and Drug Administration (FDA) was chosen as the focal point for
this project. The study conducted on the i.c inoculated TgPVR1 mouse line indicated that this mouse system could differentiate between the wild-type Leon/37 strain, the Sabin vaccine strain and a substantially neurovirulence-reverted clone of the vaccine virus isolated from a stool sample (Dragunsky et al 1993). However, the TgPVR1 mice were unable to discriminate between type-3 vaccine batches that passed or failed the MNVT (Dragunsky et al 1997). The intraspinal (IS) inoculation route proved to be no more effective than the IC route. Another mouse line, TgPVR21, was examined. This mouse line has a reduced number of transgene copies, which is shown by the lower levels of PVR mRNA and membrane associated PVR protein in the CNS compared to the TgPVR1 mice (Koike et al 1991). In the MNVT, the acceptance/rejection decision of a vaccine batch is based on the scoring of specific histological lesions in the CNS of monkeys (WHO Tech Rep 1990). By contrast, the TgPVR21 mouse neurovirulence test detected those OPV lots that failed the MNVT by evaluation of clinical signs of poliomyelitis, thus not requiring the laborious histological examination of the CNS (Dragunsky et al 1997).

A large collaborative study of type-3 OPV demonstrated, with statistical reliability, that the TgPVR21 mice provided as sensitive and reproducible a test for neurovirulence of OPV as the monkey test (Levenbook et al 2000). A statistical decision model for the acceptance/rejection decision of type-3 vaccines using the transgenic mouse test has been developed. The transgenic mouse neurovirulence test demonstrates a number of advantages over the monkey neurovirulence test:

1) Ethical and practical considerations for reduction of number of primates used in the test
2) Elimination of hazards to personnel working with the primates
3) Use of a special line of mice with highly defined genetic and microbiological quality standards
4) Significantly shorter time required for the test – 2 weeks for the mouse test instead of 1.5-2 months for the monkey test
5) Strictly objective evaluation of the test results – observation of paresis/paralysis versus semi-subjective scoring of histological lesions in the CNS of monkeys.

6) Reduction in the costs of control tests for OPV.

1.10.3 Live Mumps Vaccine Virus

In the pre-vaccine era, MuV was the leading cause of virus-induced CNS disease; a disease which has since declined due to the introduction of a mumps vaccination programme. However MuV complications in vaccinees were reported (Cizman et al 1989, Miller et al 1993). These adverse events are thought to be due to insufficient attenuation of neurovirulent vaccine virus strains. Consequently neurovirulence tests are a required part of mumps vaccine seed evaluation. However, there is a lack of a reliable animal model with which to test mumps virus neurovirulence which in turn has led to an inability to predict accurately the effect of a vaccine strain on humans, as well as hindering studies into the neuropathogenesis of MuV infection and identification of the virological determinants of neurovirulence.

Neurovirulence is measured by scoring histological lesions present in the CNS of virus inoculated monkeys. Observations by Bunyak and Hilleman in 1966 showed that this system did not always yield a valid result. The authors observed that vaccine and virulent mumps viruses often produced similar histological lesions.

Recent studies into the use of the marmoset monkey in the MNVT has yielded surprising results. Saika et al (2002) demonstrated that histological examination of the CNS of i.v inoculated marmoset monkeys could distinguish between virulent and non-virulent strains of MuV. The authors also showed that the marmosets displayed symptoms of nephritis, parotitis, pancreatitis and tonsillitis.

The severity of mumps virus histological lesions in the monkey model is evaluated using grades 1-4 (Afzal et al 1999a)

**Grade 1:** Minimal. Very mild infiltration of the subependymal zone of the ventricular wall and
very mild diffusion of the choroid plexus. Slight degree of focal dystrophic lesions as well as proliferation of the ependymal cells.

**Grade 2**: Moderate. Mild infiltration of the subependymal zone with moderate diffuse infiltration of the choroid plexus, moderate degeneration and proliferation of the ependymal cells.

**Grade 3**: Severe. Severe significant focal infiltration with more diffuse areas of dense infiltration in up to one half of the ventricular wall, subependymal oedema; severe infiltration in the choroid plexus; degeneration in some areas of the ependyma and proliferation in others with papillomatous formations.

**Grade 4**: Overwhelming. Severe inflammatory infiltration of more than one half of the ventricular wall and in the choroid plexus; loss of ependymal epithelium, adhesion of the inflamed choroid plexus to the ventricular wall.

1.10.4 Monkey Neurovirulence Test for Mumps Virus

The MNVT is required by the WHO, the EP and National Control authorities for the evaluation of the neurovirulence potency of the vaccine seed stock and the first five consecutive lots of mumps vaccines (WHO report 1994). This aims to provide assurances to the vaccine manufacturer and control authorities regarding the clinical safety of the vaccine progenitor material, although it does not ensure freedom from possible instability of virus attenuation during vaccine manufacture. There is no explicit protocol for the mumps vaccine MNVT.

Levenbook et al (1979) obtained convincing data which demonstrated a strong correlation between the MNVT for L-3 vaccine and vaccine-associated meningitis in children. In the neurovirulence test required by the WHO and the EP, the inoculum is injected into the thalamus region of mumps seronegative macaques that are then monitored 17-21 days for signs of CNS disorders or any other abnormal symptoms. At the end of the observation period, the tissues are subjected to histopathological examination. Ventricular and periventricular areas of the brain demonstrated keen histopathological responses to the
inoculation of MuV, thus the neurovirulence test was revised to concentrate assessment on these regions (Maximova et al 1996). Using the revised test, Afzal et al (1999a) examined a variety of mumps strains, both wild-type and vaccine strains, for neurovirulence. Their study concluded that the test had the ability to distinguish between wild-type and vaccine strains but not between mixed and unmixed populations or a putative neurotropic strain from its attenuated bulk precursor. Thus, ability of the test to discriminate between acceptable and unacceptable seed lots requires further examination.

1.10.5 Rat Neurovirulence Test

A rat model has been investigated as an alternative to the use of monkeys for neurovirulence testing. Viral infection during foetal development has been linked to abnormalities in the CNS and these are a major form of congenital defect in humans (Behan 1985, Brouwers et al 1995). Viral infections known to cause intrauterine or postnatal brain developmental damage include herpes simplex virus (Beers et al 1995), HIV (Brouwers et al 1995), rubella and mumps virus (Rubin et al 1998). The rat brain continues to mature after birth and is vulnerable to developmental damage following perinatal insult. It is a reasonable assumption that at this stage a neurotropic virus will incur the maximum amount of damage giving a worse case scenario of what may occur during viral infection of the human brain. The use of smaller animal models is a major consideration of modern day vivisection, but not at the cost of compromising the efficacy of the tests. By developing a model for neurovirulence on a smaller animal like the rat, or transgenic mouse as for OPV, it may be possible to improve the current test. The rat model has been shown in a number of cases with viruses to exhibit accentuated brain damage making it easily readable histologically. Rubin et al (1998) demonstrated that a neonatal rat model could distinguish between virulent and non-virulent strains of MuV. In 2000, Rubin et al suggested that the human neurovirulence of the MuV strains was proportional to the severity of the hydrocephalus in rats, but not in the lesion scores in monkeys, making the neo-natal model suitable for further evaluation.
1.11 Study Objective

Some commercial mumps vaccines have been shown to be problematic when used for large scale immunisation. The most common adverse effects reported following mumps/MMR vaccination is the development of aseptic meningitis within 8-30 days post-immunisation. All MuV vaccine strains, with the exception of the JL strain, have been attributed to post-vaccination meningitis at a variable rate. Previous work conducted at NIBSC in relation to the evaluation of the monkey model for mumps vaccine neurovirulence testing concluded the unsuitability of the model for various reasons described in previous sections. Similar conclusions were also drawn by a team working independently at the FDA in the USA. Additionally, the FDA have developed a neo-natal rat model for mumps virus neurovirulence testing that needed to be evaluated at an independent laboratory.

The current study evaluates the neo-natal rat model by using a variety of mumps virus vaccine, vaccine derived and wild-type strains, to assess its susceptibility in discriminating between strains that are predicted to have diverse neurovirulence. The majority of strains used in this study were tested in the monkey model previously and the study therefore is capable of comparing the rodent model and the primate model directly.
2 Materials and Methods

2.1 Materials

2.1.1 Cell Culture Medium and Solutions

Eagle’s Minimum Essential Medium (MEM): Sigma

0.97g MEM in 100ml distilled water pH 7.0

Penicillin and Streptomycin: 1.25g penicillin-G sodium salt, 1g streptomycin sulphate in a final volume of 100ml distilled water

Fungizone: 0.02g Fungizone in a final volume of 100ml distilled water

Bicarbonate stock: 4.4g NaHCO₃, 0.5g phenol red in a final volume of 100ml distilled water

L-Glutamine Stock: 2.9g glutamine in a final volume of 100ml distilled water

Foetal Calf Serum (FCS) Sigma

Growth Medium: 3% (v/v) bicarbonate stock
1% (v/v) penicillin and streptomycin
1% (v/v) fungizone
4% (v/v) FCS in MEM pH 7.0

Maintenance Medium: 3% (v/v) bicarbonate stock
1% (v/v) penicillin and streptomycin
1% (v/v) fungizone
1% (v/v) FCS
<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration/Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>1.07g NaCl, 0.03g KCl, 0.017g CaCl₂·2H₂O, 0.013g MgCl₂·6H₂O, 0.40g Na₂HPO₄·12H₂O, 0.03g KH₂PO₄</td>
</tr>
<tr>
<td></td>
<td>in a final volume of 100ml distilled water pH 7.4</td>
</tr>
<tr>
<td>Trypsin stock:</td>
<td>Sigma 6mg trypsin in 100ml saline solution</td>
</tr>
<tr>
<td>EDTA stock:</td>
<td>0.4g EDTA in a final volume of 100ml saline solution</td>
</tr>
<tr>
<td>Trypsin stock:</td>
<td>1ml trypsin stock, 1ml EDTA stock, 1ml bicarbonate stock in 100ml saline</td>
</tr>
<tr>
<td>Carboxymethyl Cellulose (CMC):</td>
<td>Sigma 3.2g CMC in 100ml PBS, 2ml penicillin and streptomycin, 2ml fungizone, 8ml FCS, 10ml bicarbonate stock in a final volume of 220ml MEM</td>
</tr>
</tbody>
</table>
Methyl Violet Stain: 2.5g methyl violet stain, 100ml ethanol, in a final volume of 500ml distilled water

2.1.2 Histological Reagents

Formol-saline 40% (v/v) formaldehyde (Sigma), 9 g sodium chloride [BDH] made up to 100ml with distilled water

Albumin Preparation Glycerin (Sigma) was added 1:1 to a liquid egg white and filtered through muslin.

Gallocyanin Stain 5g Chromic potassium sulphate, 150mg gallocyanin in a final volume of 1200ml distilled water.

2.1.3 Virus Strains

LO-1 (Mu90): Mumps virus wild type strain. Isolated from a male child in 1988 exhibiting symptoms of parotitis. The virus was isolated from the saliva sample on Vero cells at NIBSC.

JL (MSD) Vaccine Bulk: Mumps vaccine bulk of the Jeryl Lynn strain was obtained from Merck Sharpe and Dohme. The vaccine has been shown to contain two distinct variants designated JL2 and JL5.

JL (SKB) Vaccine Bulk: Mumps vaccine bulk of the Jeryl Lynn strain was obtained from SmithKline Beecham. The seed stock component of this vaccine was derived from the JL5 component of the mixed MSD JL vaccine.

Urabe Vaccine Bulk: Mumps vaccine bulk of the Urabe strain was obtained from SmithKline Beecham.
Lo4/2: A post-vaccine strain that was isolated from a vaccine recipient who had received the Urabe vaccine but who did not exhibit any symptoms. The isolate has been passaged twice on the Vero cell line.

Nt5/2: A post-vaccine strain that was isolated from a Urabe MMR vaccine recipient who developed aseptic meningitis. This isolate has been passaged twice on Vero cells.

JL2/4: A sub-component of the JL MSD vaccine that was plaque purified in 1990. This sample was subsequently passaged on Vero cells four times.

JL5/4: A sub-component of the JL MSD vaccine that was plaque purified in 1990. This sample was subsequently passaged on Vero cells four times.

Clinical Material: 99-0333 A wild-type strain of mumps virus. This is unpassaged clinical material, saliva, that was originally derived from a 9 year old female with symptomatic mumps.

Clinical Material: 99-0333P1/V This sample was produced by passaging the above specimen, 99-0333, once on Vero cells.

2.2 Virus Propagation

Viruses were propagated using the following technique:

Growth medium was aspirated from a 25cm² tissue culture flask containing an 80% confluent cell monolayer of Vero cells (approximately 5x10⁶ cells) and the appropriate dilution of the virus inoculum, in maintenance medium, was added. The dilution of virus inoculum used was initially $3\log_{10} \text{TCID}_{50}/\text{ml}$ of virus, giving an approximate multiplicity of infection (MOI) of 0.0002 (1 TCID₅₀ per 5000 cells). The cells were incubated with virus for
approximately 2 hours in a humidified incubator at 35°C, 5% CO₂, after which the inoculum was aspirated and fresh maintenance medium added. The infected cells were incubated at 35°C, 5% CO₂ until 80% CPE was observed. If no CPE was observed then the infected cell sheets were harvested 10 days post infection.

Virus was harvested by freezing the tissue culture flask containing infected cells at -70°C and thawing at room temperature to release any cell bound virus. The cell debris was removed from the virus by centrifugation at 1600 x g for 10 minutes at 25°C. The supernatant fluid, containing virus, was aliquoted and stored at -70°C.

2.3 Inocula Titration and Virus Titres

The virus inocula to titrated were done so in an Infectivity Assay in order determine viral titres.

2.3.1 Infectivity Assays

Infectivity assays were used in order to determine the titre of virus in a given sample. This was achieved by using various dilutions of the sample to infect cells in a 24 well plate and counting the number of plaques after a 7 day incubation period, assuming that one plaque is equivalent to one infectious virus particle.

Log dilutions of the viruses were made in MEM (Section 2.1) and 50µl volumes were added in duplicate to a 24 well plate. A Vero cell suspension was prepared in 40ml MEM (Section 2.1) from one 100cm² flask. One millilitre of the cell suspension was added to each well and incubated at 35°C in 5% CO₂ for three hours. All the medium was removed and 1ml of carboxymethyl cellulose (CMC) [Section 2.1]) medium was added to each well. The plates were then incubated at 35°C in 5% CO₂ for one week. The CMC layer was removed by suction pipetting into chloros. The cell sheet was washed with PBS and stained for 30 minutes with 0.5% methyl violet stain in 20% ethanol.

2.3.2 Infectivity Assay Analysis

The assay plates were placed underneath a lighted magnifying glass. The dilution of the
virus which produced between 50-200 plaques per well was chosen to be counted in order to reduce any error that may occur due to the counting of overcrowded plaques. After the number of plaques had been counted and recorded, the number of plaques in 1ml of the original inoculum was worked out by the calculation below.

\[
\text{Calculation: } \frac{N \times \text{DF}}{\text{TV}} \times 1000
\]

Where

\[N = \text{Number of plaques counted}\]

\[\text{DF} = \text{Dilution factor}\]

\[\text{TV} = \text{Volume of virus solution in each well (µl)}\]

This calculation gives the amount of virus in pfu/ml of the stock sample.

2.4 Rat Inoculation

After determination of the viral titres, pregnant Lewis rats were purchased to arrive at NIBSC during days 15-17 of the gestation period. The viruses to be inoculated were thawed and diluted to a concentration of 400 pfu/ml in MEM. Each virus strain was inoculated into a single litter of newborn pups, not older than 24 hours. For this a single pup was placed in a biosafety cabinet on several layers of paper towels. Five hundred micro-litres of the viral inocula was drawn up into a 2ml syringe and a 27G needle was attached. The pup was grasped firmly behind the head with the thumb and forefinger of one hand. Using the other hand, the needle was inserted through the left parietal area of the skull approximately 1-2mm left of the midline and approximately 2-3mm caudal to an imaginary line drawn midway between eyes and the ears, Figure 2.1.

The needle penetrated into the brain no more than 3mm below the outside surface of the head, and approximately 25µl of inocula (100 pfu of virus) was slowly injected. The inoculated pup was returned to its cage before any other pups were inoculated. The same syringe and needle were used to inoculate all the pups in the same litter. Once a litter of
pups had been inoculated, the syringe, needle and paper towels were discarded and replaced, gloves were changed and the next litter to be inoculated was placed in the biosafety cabinet.

The rats were observed daily throughout the 30 day duration of the experiment for any abnormalities such as CNS abnormalities, severe motor abnormalities, inability to access water and food and wasting disease, as well as any pain or distress.

2.5 Extraction of Tissue

Rats to be euthanised were placed in a sealed container and exposed to carbon dioxide ($\text{CO}_2$). After death, the rats were decapitated with a large pair of scissors by cutting at the base of the skull through the brain stem. The skin was removed from the head and, using rongeers, the upper part of the skull removed in order to expose the whole brain. The brain was removed from the skull by gently prying it from the calvarium with a closed pair of fine forceps. The brain was placed immediately in 10% formalin solution and fixed at 4°C for 1-2 weeks.

2.6 Histology

Classic histological techniques, described below, were used to identify microscopic changes in tissue samples.

2.6.1 Cassette Labelling

Yellow plastic histology cassettes (R A Lamb) were mounted into an RA Lamb Cassette MicroWriter Machine. Using the MWC computer programme, the cassettes were labelled with the corresponding animal numbers (M02) which were logged in the Miscellaneous Histology Book. These were placed in order ready to be used.

2.6.2 Dissecting brain samples

Whole brain tissue samples were removed from the formol-saline and placed on a dissection board. Using a disposable eight inch trimming knife (RA Lamb), the brain was cut into two by cutting down the centre of the brain.
Figure 2.1: Location of the Inoculation Site. Showing the desired location of the needle for injection of the MuV into the neo-natal rat brain.
The central part of the brain was removed and replaced in the formol-saline container. The remaining left and right hand side of the brains are trimmed as shown in Figure 2.2, cuts 3 and 4. The tissue was placed with the side closest to the centre of the brain down in the previously marked cassettes (Section 2.7.1). A plastic lid (R A Lamb) was secured onto the cassette and placed in a large plastic box containing formol-saline where it remained until the brain samples were ready for processing.

2.6.3 Processing of Brain Samples

The tissue samples were placed in a Leica TP 1050 Tissue Processor and run on a neurovirulence cycle with the following features as described in Table 2.1.

2.6.4 Sample Blocking

The samples were removed from the Leica TP 1050 Tissue Processing Machine and placed immediately into the molten paraffin wax hold of the wax dispenser (RA Lamb Blockmaster III). The lid from a cassette in the molten paraffin wax was removed carefully. The sample was removed from the cassette and placed in a small metal sample tray containing a layer of molten wax. This was then placed on the small metal freezing plate of the Blockmaster III and frozen so that the sample remained in place. The yellow plastic cassette, with M02 number, was placed over the small metal sample tray and filled with molten wax. The metal tray was placed on the large freezing plate to set. This was repeated for all samples. Once they had set, the metal trays were removed by snapping them off. The embedded samples were sorted according to their numbers ready for sectioning.

2.6.5 Slide Labelling and Preparation

Menzel Superfrosted Slides were labelled with the corresponding cassette number using an RA Lamb MicroWriter. Slides were stacked into the entry port and using the MWC computer programme, sample numbers were etched onto the slides in duplicate. To aid sample adherence to the slide a preparation of albumin was spread over the slides using a finger.
Table 2.1: Neurovirulence cycle for processing of rat brain tissue.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Time (hours)</th>
<th>Temperature °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formalin</td>
<td>00.05</td>
<td>Ambient</td>
</tr>
<tr>
<td>Industrial Methylated Spirits (IMS) 50% Concentration</td>
<td>01.00</td>
<td>Ambient</td>
</tr>
<tr>
<td>IMS (70%)</td>
<td>01.00</td>
<td>Ambient</td>
</tr>
<tr>
<td>IMS (95%)</td>
<td>01.00</td>
<td>Ambient</td>
</tr>
<tr>
<td>IMS (100%)</td>
<td>01.00</td>
<td>Ambient</td>
</tr>
<tr>
<td>IMS (100%)</td>
<td>01.00</td>
<td>Ambient</td>
</tr>
<tr>
<td>Ethanol (50%) Xylene (50%)</td>
<td>02.00</td>
<td>Ambient</td>
</tr>
<tr>
<td>Xylene</td>
<td>02.00</td>
<td>Ambient</td>
</tr>
<tr>
<td>Xylene</td>
<td>02.00</td>
<td>Ambient</td>
</tr>
<tr>
<td>Paraffin Wax</td>
<td>02.00</td>
<td>60</td>
</tr>
<tr>
<td>Paraffin Wax</td>
<td>02.00</td>
<td>60</td>
</tr>
<tr>
<td>Paraffin Wax</td>
<td>02.00</td>
<td>60</td>
</tr>
</tbody>
</table>
2.6.6 Microtomy

Brain samples were placed in a Leica RM2135 Microtome set at 20μm trimming thickness. Excess wax was trimmed from the embedded tissue until the sample was reached. The sample block was removed and placed sample down in a layer of Mollifex (BioRad) for 20 minutes in order to soften the tissue for cutting. Once softened, the blocks were re-mounted onto the microtome set at 12μm cutting thickness. Once two or three sample slices had been obtained, they were floated on water in a water bath set at 42°C in order to flatten the samples and remove creases. Once the samples were flat, slides were immersed in the water beneath the floating sections and removed carefully onto the slide. These slides were then placed in a 36°C oven to dry. The paraffin wax blocks were stored in large metal storage tray containers where they can be kept indefinitely.

2.6.7 Staining

Once the slides had been allowed to dry, at least overnight, they were de-waxed. The slides were placed in Xylene (BDH) for 5 minutes, removed and placed in a fresh container of xylene for 5 minutes. This was repeated until visible examination revealed that all wax had been removed. The slides were then dipped in Celloidin, washed with distilled water, placed in a Gallocyanin stain container and incubated at 56°C for 3 hours, after which the slides were removed and washed gently in distilled water. The slides were then placed in increasing concentrations of ethanol every five minutes starting with 70%, 90%, then 100%. After the final 100% ethanol soak, the slides were placed in xylene for 5 minutes and then re-placed in another container of xylene. Individual slides were taken from the xylene and placed face down on blotting paper whilst the back was dried with a white Wypall. The slides were turned over and using a plastic Pasteur pipette, two large spots of DPX (Sigma) were placed on the sample which were then covered with a glass coverslip making sure that the DPX was spread completely over the sample and that no bubbles appeared. If bubbles appeared, they were removed by gently pressing the coverslip to move the bubbles to the
edge of the slide. The slide was then left to dry overnight to secure the coverslip to the slide. Once dried, any leaked dry DPX was removed from around the coverslip carefully using a scalpel blade. The slide was then ready for microscopic examination.

2.7 Microscopy

Once the slides had been prepared, as in the previous section, they were arranged according to their MO2 number in slide sample holders ready for microscopic examination with a Zeiss microscope at a magnification of x100.

2.7.1 The Cerebral Cortex

The cerebral cortex is located above the horse-shoe shaped hippocampus. Using a graticule placed in the microscope lens, the width of the cerebral cortex from the top of the hippocampus to the edge of the cortex was measured, as shown in Figure 2.2. This measurement was recorded for each slide.

2.7.2 The Third Ventricle

The third ventricle is located at the lower edge of the dorsal horse-shoe (Ammon's horn) of the hippocampus (Figure 2.3). First, the ventricular ependyma was analysed for any evidence of cellular migration or roughness. Then the sub-ependyma was analysed for evidence of oedema surrounding the ventricle. Lastly the size of the third ventricle was measured using a graticule as shown in Figure 2.3 and noted. Also, a grading on the size of the third ventricle was made by eye examination and recorded along with the graticule measurement.
Figure 2.2: Cerebral Cortex. The arrow details the measurements taken into consideration when scoring the cerebral cortex.
Figure 2.3: Third ventricle. The arrow details the area of the third ventricle taken in to consideration when scoring.
2.7.3 The Lateral Ventricle

The lateral ventricle is located at the upper edge of the dentate gyrus of the hippocampus (Figure 2.4). First, the ventricular ependyma was analysed for any evidence of cellular migration or roughness. Then the sub-ependyma was analysed for evidence of oedema surrounding the ventricle. Lastly the size of the lateral ventricle was measured using a graticule as shown in Figure 2.4 and noted. In addition, a grading on the size of the lateral ventricle was made by eye examination and recorded along with the graticule measurement.

2.7.4 The Cerebellum

The cerebellum is a highly folded structure as can be seen in Figure 2.5. There are three layers to the cerebellum, the external molecular layer, the Purkinje cell layer and the internal granular layer (Figure 2.6) on the extent of visible damage.

2.7.5 Slide storage

Once the slides had been read, they were stored in metal slide storage cabinets at room temperature, where they can be stored.
Figure 2.4: The Location of the Lateral ventricle. The arrow details the area of the lateral ventricle taken in to consideration when scoring.
Figure 2.5: The cerebellum: defining the three layers. The arrows show the three layers of the cerebellum.
2.8 FDA Scoring Method

This method only takes into consideration the size of the lateral ventricle in relation to the rest of the brain.

A grid of squares measuring 1mm by 1mm was placed over a neo-natal rat brain exhibiting hydrocephalus. The number of squares that the lateral ventricle encompassed was measured and noted (a). The number of squares the whole of the brain (excluding the brain stem) was also measured (b). (a) was then divided by (b) and the resulting figured multiplied by 100 to give the percentage area the lateral ventricle occupies of the whole brain. This percentage was then noted.
3 Results

3.1 Introduction

To assess and validate the neo-natal rat as a model for mumps neurovirulence, newborn rats were inoculated i.c with wild-type and vaccine strains of mumps virus and assessed by histopathological grading of ten areas of the brain as follows:

- Cerebral Cortex Thickness
- Third Ventricle:
  - Epithelial Lining
  - Sub-epithelial Area
  - Size
- Lateral Ventricle:
  - Epithelial Lining
  - Sub-epithelial Area
  - Size
- Cerebellum:
  - Molecular Layer
  - Purkinje Cell Layer
  - Granular Layer

Rubin et al (1998a) have demonstrated that variation in the size of the lateral ventricle could be indicative of the neurovirulent activity of mumps virus in a neonatal rat brain. Infection by MuV of the ventricular ependyma alters the equilibrium flow of cerebrospinal fluid (CSF) into the ventricular space, increasing the size of the ventricle. For this reason, the variation in the size of the lateral ventricle area of the brain has been proposed as a suitable marker to determine the levels of neurovirulence that may be associated with a particular strain or vaccine preparation.

In addition to the ventricle size other parameters, listed above, were also assessed during this study in order to identify what other areas of the brain were affected following I.C inoculation of mumps virus. If altered following inoculation they could provide additional histopathological markers for the assessment of mumps virus neurovirulence.

It has been noted in poliovirus neurovirulence studies of macaques, undertaken at NIBSC,
that the thickness of the cerebral cortex alters with the degree of neurovirulence.
Observations made during this project propose that a decrease in the thickness of the cerebral cortex is a direct result of an increase in the size of the lateral ventricle, Appendix II. Rubin et al (1998) noted that an increase in size of the third ventricle accompanied the increase in size of the lateral ventricle; however, it was observed that the third ventricle size increase was not as significantly large as the lateral ventricle increase. As MuV infects the cell lining of the ventricular space, it was thought probable that there might be some detectable changes in cellular morphology and distribution. The molecular, purkinje and granular layers of the cerebellum were also considered as suitable areas for measuring the neurovirulent activity of MuV, as Rubin et al (1998) described the occurrence of cellular migration in MuV infected neo-natal rat brain samples.

By studying the parameters listed above, the usefulness of the neo-natal rat to detect neurovirulence of mumps virus strains would be established.
In each RNVT, samples were designated a number or letter for identification. The list of viruses inoculated and the number of animals used in each RNVT is shown in Table 3.1. The complete histopathological data of each animal for all RNVT are tabulated in Appendix 1.
Table 3.1: List of RNVT with viruses and number of animal inoculated.

<table>
<thead>
<tr>
<th>RNVT</th>
<th>Mumps Viruses Used</th>
<th>Designation</th>
<th>No of Animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>Lo1/Mu90 Wild Type</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>SKB JL Vaccine Bulk</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>SKB Urabe Bulk Vaccine</td>
<td>3</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>Clinical Material</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Clinical Material 1V</td>
<td>11</td>
<td>4</td>
</tr>
<tr>
<td>D</td>
<td>Lo1/Mu90 Wild Type</td>
<td>1</td>
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</tr>
<tr>
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<td>SKB JL Vaccine Bulk</td>
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<td></td>
<td>Ni5 Urabe Virus</td>
<td>7</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>FDA C</td>
<td>C</td>
<td>22</td>
</tr>
<tr>
<td>E</td>
<td>Lo1/Mu90 Wild Type</td>
<td>1</td>
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<td>SKB JL Vaccine Bulk</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>MSD JL Vaccine Bulk</td>
<td>4</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>FDA D</td>
<td>D</td>
<td>21</td>
</tr>
<tr>
<td>F</td>
<td>Lo1/Mu90 Wild Type</td>
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<td>12</td>
</tr>
<tr>
<td></td>
<td>SKB JL Vaccine Bulk</td>
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<td>10</td>
</tr>
<tr>
<td></td>
<td>Lo4/2 Urabe Virus</td>
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</tr>
<tr>
<td></td>
<td>FDA A</td>
<td>A</td>
<td>19</td>
</tr>
<tr>
<td>G</td>
<td>Lo1/Mu90 Wild Type</td>
<td>1</td>
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<td>SKB JL Vaccine Bulk</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>JL2/4 Sub-species</td>
<td>8</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>JL5/4 Sub-species</td>
<td>9</td>
<td>19</td>
</tr>
<tr>
<td>H</td>
<td>Lo1/Mu90 Wild Type</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>SKB JL Vaccine Bulk</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>FDA E</td>
<td>E</td>
<td>20</td>
</tr>
<tr>
<td>I</td>
<td>Lo1/Mu90 Wild Type</td>
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<td>11</td>
</tr>
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<td></td>
<td>SKB JL Vaccine Bulk</td>
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<td>11</td>
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<tr>
<td></td>
<td>FDA B</td>
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<td>23</td>
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<tr>
<td>J</td>
<td>Lo1/Mu90 Wild Type</td>
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<tr>
<td></td>
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<td>9</td>
</tr>
<tr>
<td></td>
<td>Diluent</td>
<td>5</td>
<td>19</td>
</tr>
</tbody>
</table>
3.2 Histological Grading Procedures.

The grading method used at NIBSC is based on a visual evaluation of the specified parameters. These were classified into a grading, as below, depending on the severity of the change.

Table 3.2: Summary of the grading system used by NIBSC

<table>
<thead>
<tr>
<th>Grading</th>
<th>Severity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative (--)</td>
<td>No difference from the control</td>
</tr>
<tr>
<td>Low-Level (-/+)</td>
<td>Marginal increase in lateral ventricle size from the control.</td>
</tr>
<tr>
<td>One (+)</td>
<td>Mild. Noticeable increase in lateral ventricle size.</td>
</tr>
<tr>
<td>Two (++)</td>
<td>Moderate. Increase in size of lateral ventricle.</td>
</tr>
<tr>
<td>Three (+++)</td>
<td>Severe. Lateral ventricle covers a large part of the brain.</td>
</tr>
<tr>
<td>Four (++++)</td>
<td>Overwhelming. Lateral ventricle covers most of the brain.</td>
</tr>
</tbody>
</table>
3.2.1 Negative Grading

Animals inoculated with the sample diluent (ie: MEM only) did not show any increase in the lateral and third ventricles (Figure 3.1A). The cerebral cortex showed no alteration in its thickness (Figure 3.1B). The epithelial layers of each ventricle were smooth and showed no evidence of cell migration or surrounding oedema (Figure 3.1C and D). No cellular migration or clumping occurred in the layers of the cerebellum (Figure 3.1E).

3.2.2 -/+ grading

Animals displaying a low-level affect showed a small visible increase in the size of the lateral ventricle, whilst the third ventricle showed no increase in size (Figure 3.2A). There was no evident decrease in the thickness of the cerebral cortex (Figure 3.2B). The ventricular epithelium gave a slight roughened appearance though no oedema was visible (Figure 3.2C and D). The cerebellum showed no evidence of cellular migration within any of the three layers (Figure 3.2d).
Figure 3.1: Examples of sections of the brains of animals inoculated with the diluent. Showing the third and lateral ventricle sizes (A), the cerebral cortex thickness (B), the epithelial and sub-epithelial layer of the lateral ventricle (C), the epithelial and sub-epithelial layers of the third ventricle (D) and the three layers of the cerebellum (E)
Figure 3.2: Examples of sections of the brains of animals showing a typical low-level effect. Showing a small increase in the size of the lateral ventricle and no increase in the third ventricle (A), a decrease in the thickness of the cerebral cortex (B), the epithelial and sub-epithelial layers of the lateral ventricle (C), the epithelial and sub-epithelial layers of the third ventricle (D) and the three layers of the cerebellum (E).
3.2.3 Grade One (+)

In animals displaying a Grade One effect, the lateral and third ventricles showed an increase in size in comparison to the negative control group (Figure 3.3A). The thickness of the cerebral cortex decreased (Figure 3.3B), and the ventricular epithelial lining showed roughened edges and cellular migration, with surrounding oedema (Figure 3.3C and D). The cerebellum molecular layer showed cell migration towards the centre of the layer, with the remaining two layers being unaffected (Figure 3.3E).

3.2.4 Grade Two (++)

In neo-natal rat brains displaying Grade Two activity, the lateral and third ventricles were observed to have increased in size, compared to a Grade One neurovirulent activity (Figure 3.4A). The ventricular epithelial of the third ventricle showed an increase in cell migration though no sample showed any evidence of oedema at Grade Two (Figure 3.4C). The lateral ventricle showed an increased level of cellular migration and oedema surrounding the ventricle. The cerebral cortex displayed a marked decrease in thickness (Figure 3.4B), and the molecular layer of the cerebellum showed evident migration of cells towards the centre of the layer with noticeable clumping (Figure 3.4D).

Occasionally, a sample shows evidence of activity which cannot be unambiguously classified either as a Grade One or Two but may fall between two grades. Such samples were graded as (+/++). This was more common for the increase in size of the lateral ventricle as shown above, but was occasionally also observed in other areas, such as the thickness of the cerebral cortex, the lateral ventricular epithelial and sub-epithelial layers.
Figure 3.3: Examples of sections of the brains of animals showing a typical Grade One effect. Showing an increase in the size of the lateral ventricle (a), the decrease in the cerebral cortex thickness (B), the epithelial and sub-epithelia layers of the lateral ventricle (C), the epithelial and sub-epithelial layer of the third ventricle (D), and the three layers of the cerebellum (E)
Figure 3.4: Examples of sections of the brains of animals showing a typical Grade Two effect. Showing an increase in the size of the lateral and third ventricle (A), a decrease in the cerebral cortex thickness (B), the epithelial and sub-epithelial layer of the third ventricle (C), the epithelial layer of the lateral ventricle (D), and the three layers of the cerebellum (E)
3.2.5 Grade Three (+++)

Rat brains which displayed a Grade Three effect showed an increase in size of the lateral and third ventricles in comparison to Grade Two (Figure 3.5A). The cerebral cortex showed a decrease in size in comparison to Figure 3.4 (Figure 3.5B). There was no histopathological evidence of epithelial or sub-epithelial changes of Grade Three activity for the lateral and third ventricle. The cerebellum showed an increase in cellular migration and clumping in the molecular layer in comparison to Grade Two, and there was also evidence of cellular bunching in the Purkinje cell layer. The granular layer showed no change (Figure 3.5 C and D). The specimens that showed higher activity than Grade Two but lower than Grade Three were classified as (++/+++); this was only found in the size of the lateral ventricle.

3.2.6 Grade Four (++++)

Of the few animals that displayed Grade Four activity, it was presented only in the size of the lateral ventricle, the thickness of the cerebral cortex and the cerebellum regions. The size of the lateral ventricle had increased markedly from a Grade Three scale (Figure 3.6A); the thickness of the cerebral cortex had also decreased markedly (Figure 3.6B). The clumping of cells in the molecular layer of the cerebellum was evidently increased (Figure 3.6C), and the cells in the Purkinje layer had bunched and pinched off into the granular layer which showed evidence of oedema-like cellular damage (Figure 3.6D).
Figure 3.5: Examples of sections of the brains of animals showing a typical Grade Three effect. Showing an increase in the size of the lateral ventricle (A), a decrease in the size of the cerebral cortex (B) and the migration of the cells of the molecular layer of the cerebellum (C + D)
Figure 3.6: Examples of sections of the brains of animals showing a typical Grade Four effect. Showing an increase in the size of the lateral ventricle (A), a decrease in the thickness of the cerebral cortex (B), the clumping of cells in the molecular layer (C), and the clumping of purkinje cells in the granular layer (D).
3.3 Control Viruses

In each of the eight RNVT's performed in this study, two strains of MuV were used as experimental controls. Lo1/Mu90 was used as a positive neurovirulent control and SKB JL vaccine strain was used as a negative control. Lo1/Mu90 MuV is a wild type strain which was isolated from an individual exhibiting parotitis. This virus has been used before in macaque studies undertaken at NIBSC which concluded that amongst the viruses tested the Lo1/Mu90 was the most neurovirulent strain (Afzal et al 1999a). It was therefore selected to be used as a positive control in this study. The SKB JL MuV vaccine strain has not been reported to be associated with severe post-vaccination adverse events in recipients and was also used in macaque studies at NIBSC, in which this virus was found to have no neurovirulent effect.

Each RNVT experiment contained, in addition to the test samples, a positive control and a negative control. The cumulative data shown in Table 3.3 for these controls can be used to identify discrepancies between individual tests. The data show assay-to-assay variation between different RNVT's which were conducted at different times due to logistical and technological reasons. Based on the previous results of the monkey model, it was expected that SKB JL Vaccine Bulk inoculum would show no evidence of neurovirulence. Analysis of Table 3.3 reveals that in each test about 40-90% of animals inoculated with SKB JL preparation showed no signs of enlargement of the lateral ventricle. Most of the remaining animals could not be conclusively scored by the histopathological parameters applied as whether or not the virus had any effect and were, therefore, classified into the (-/+ ) grade. However, some animals in the RNVT D and I exhibited up to a Grade One (9% for D [2 animals] and 18% for I [4 animals]), and Grade Two (9% for I [2 animals]) level effects contributing to a higher background scoring profile than other RNVTs.
Table 3.3: Summary of the data generated for the size of the lateral ventricle of the positive and negative control viruses in each RNVT

<table>
<thead>
<tr>
<th>Grading</th>
<th>B</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>I</th>
<th>J</th>
<th>Average %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pos²</td>
<td>Neg</td>
<td>Pos</td>
<td>Neg</td>
<td>Pos</td>
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<td>Pos</td>
<td>Neg</td>
<td>Pos</td>
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<td>-</td>
<td>-</td>
<td>50</td>
<td>-</td>
<td>54.5</td>
<td>-</td>
<td>40</td>
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<tr>
<td>-/+</td>
<td>10²</td>
<td>37.5</td>
<td>10</td>
<td>36.4</td>
<td>-</td>
<td>60</td>
<td>8.3</td>
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<td>45.5</td>
</tr>
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<td>+</td>
<td>30</td>
<td>12.5</td>
<td>60</td>
<td>9</td>
<td>-</td>
<td>-</td>
<td>33.4</td>
<td>-</td>
<td>40</td>
</tr>
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<td>+/-</td>
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<td>-</td>
<td>10</td>
<td>-</td>
<td>16.7</td>
<td>10</td>
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<td>11.1</td>
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<td>10</td>
<td>-</td>
<td>41.7</td>
<td>40</td>
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<td>22.2</td>
</tr>
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<td>+++/</td>
<td>10</td>
<td>-</td>
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<td>10</td>
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<td>-</td>
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<td>+++</td>
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<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

² the positive (Pos) control is Lo1-Mu90 and the negative (Neg) control is SKB JL Vaccine.
² the data are expressed as the percent of animals that score a particular grade.
In the macaque neurovirulence model, the Lo1/Mu90 strain scored higher compared to other strains evaluated. Analysis of the Lo1/Mu90 strain by the RNVT (Table 3.3) shows differences between various tests. The assessment of this virus group spread mainly between Grade One and Grade Two amongst the difference RNVT's, and is therefore considered normal for this virus group. For RNVT B, grading for the positive control ranges from (-/+) to Grade Three, which is the widest range. Approximately 70% of the specimens of RNVT E are in the Grade Three to Grade Four range which is not reflected in the scoring of the positive control samples of other RNVT's. This relatively high scoring for Mu90 in this RNVT is not observed with animals that were inoculated with the negative control strain.

3.3.1 Control Parameters

Table 3.3 shows data generated for the size of the lateral ventricle of the positive and negative controls exclusively. The results of the other study parameters referred to in Section 3.1 are shown graphically in Figure 3.7 for the positive control Lo1/Mu90 and in Figure 3.8 for the negative control JL SKB vaccine.

The collective data from all animals inoculated with the positive control preparation from each RNVT shows that the thickness of the cerebral cortex could be graded from Negative to Grade Two (Figure 3.7). The Third Ventricle data showed that 60-75% of animals scored negative with the remainder scoring up to Grade One. Occasionally a grade higher than One was seen (Figure 3.7). The lateral ventricle was the most affected area of the brain with the majority of specimens showing Grade One level effects in each parameter assessed. The molecular layer of the cerebellum also showed a variety of grades from Negative to Grade Two/Three, again with most scoring at a Grade One (Figure 3.7). Both the Purkinje and Granular Cell Layers scored mainly negative, although the Purkinje cell layer had one specimen graded to a level three.
Figure 3.7: Grading assessment of the ten parameters of animals inoculated with the LO1/Mu90 strain.

Key:
1. Thickness of cerebral cortex
2. Third Ventricle; Epithelial changes
3. Third Ventricle; Sub-epithelial changes
4. Third Ventricle; Size
5. Lateral Ventricle; Epithelial changes
6. Lateral Ventricle; Sub-epithelial changes
7. Lateral Ventricle; Size
8. Cerebellum; Molecular layer
9. Cerebellum; Purkinje cell layer
10. Cerebellum Granular layer
Figure 3.8: Grading assessment of the ten parameters of animals inoculated with SKB JL Vaccine strain.

Key: 1 Thickness of the Cerebral Cortex
2 Third Ventricle; Epithelial changes
3 Third Ventricle; Sub-epithelial changes
4 Third Ventricle; Size
5 Lateral Ventricle; Epithelial changes
6 Lateral Ventricle; Sub-epithelial changes
7 Lateral Ventricle; Size
8 Cerebellum; Molecular layer
9 Cerebellum; Purkinje cell layer
10 Cerebellum Granular layer
The collective data of various parameters of animals inoculated with the negative control virus in various RNVT’s show that the cerebral cortex remains unchanged in the majority of specimens, with only two specimens showing Grade one level effects (Figure 3.8). The virus exhibits no effects on the Third Ventricle. There was a small effect on the lateral ventricle. The epithelial layer showed slight signs of infection with one specimen scoring Grade One, as did the surrounding area, with four specimens of Grade One. The ventricle size increased in three specimens on average to Grade One and one specimen to Grade Two. Two specimens showed the virus having an effect on the molecular layer of the cerebellum to a Grade One, with the remainder of the specimens showing a negative or low-level effects (Figure 3.8). The Purkinje cell layer showed two specimens having a low-level effect with the Granular layer showing no effects.

Both Lo1/Mu90 and SKB JL Vaccine Bulk support the observation that the lateral ventricle and the cerebellum appear to be the regions of the brain which are affected most following MuV I.C inoculation (Figure 3.8). The third ventricle exhibits some change in response to Lo1/Mu90 but the change is less significant than the one demonstrated by the lateral ventricle, and therefore would appear to be a less suitable indicator of neurovirulence in the rat model. Similarly the cerebral cortex thickness shows a variety of responses and does not appear to be a reliable parameter for measuring the neurovirulence mumps virus strains.

3.3.2 Observations of Jeryl Lynn Mumps Vaccine Inoculated Animals

For the purpose of this report, data presented for viruses under test are grouped together in relation to their genetic similarities and not according to the groupings used to conduct RNVT’s (Appendix I). This section details the results of mumps viruses that were derived from the Jeryl Lynn vaccine strain (MSD). These viruses were studied in two different RNVT’s i.e:

- RNVT E - MSD JL Vaccine Bulk
- RNVT G - JL2/4 Sub-species
- RNVT G - JL5/4 Sub-species
Table 3.4: Grading assessment of the Lateral Ventricle size for the JL vaccine and its associated derivatives.

<table>
<thead>
<tr>
<th>Grading</th>
<th>MSD JL Vaccine Bulk</th>
<th>Percentage of controls for RNVT E</th>
<th>JL2/4 Subspecies</th>
<th>JL5/4 Subspecies</th>
<th>Percentage of controls for RNVT G</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>-</td>
<td>100</td>
<td>0</td>
<td>40</td>
<td>63.1</td>
<td>5.3</td>
</tr>
<tr>
<td>-/+</td>
<td>0</td>
<td>0</td>
<td>60</td>
<td>31.6</td>
<td>68.4</td>
</tr>
<tr>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5.3</td>
<td>10.5</td>
</tr>
<tr>
<td>+/-</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>15.8</td>
<td>10</td>
</tr>
<tr>
<td>++</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>40</td>
</tr>
<tr>
<td>++/+</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+++</td>
<td>0</td>
<td>40</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>++++</td>
<td>0</td>
<td>30</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Average %</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

89
The MSD JL Vaccine Bulk virus had no effect on the size of the lateral ventricle. The SKB JL Vaccine Bulk virus, used as a negative control, appears to have more effect than the MSD JL Vaccine Bulk. The data of Lo1/Mu90 strain, Table 3.3 and Figure 3.7, show that Grade One to Grade Two level effects were the normal effect of the positive control preparation. In RNVT E the higher assay background may be attributable to an anomaly in the slide reading or in the tissue processing (Tables 3.3 and 3.4). However, this is unlikely since the negative control results of this test did not score unusually high.

The JL2/4 vaccine sub-component virus does not exhibit a significant effect on the lateral ventricle in comparison to the negative control in RNVT G, although it did have one specimen scored in the Grade One group.

The JL5/4 vaccine sub-component virus exhibits more effect on the lateral ventricle in comparison to the negative control. Only one specimen showed no effect, with a large majority of specimens exhibited relatively low-level effects. Two specimens had Grade One and three specimens had Grade Two level effects. In comparison to the negative control, the JL5/4 vaccine sub-variant had a more enlarging effect on the lateral ventricle.

The scoring results of animals inoculated with positive control virus in RNVT E and RNVT G showed a majority of specimens were graded between Grade One and Two, whereas the animals inoculated with the negative control virus were mostly divided between negative and -/+ groups, in line with the distribution seen in Table 3.3 and Figure 3.8.

There is a difference in the neurovirulence of the two virus sub-components of the MSD JL Vaccine Bulk, as 5.3% specimens of animals inoculated with JL5/4 were found to be completely negative compared to 63.1% of animals inoculated with JL2/4. However MSD JL Vaccine Bulk virus had no effect on the lateral ventricle as all specimens were negative.

In relation to other parameters discussed in section 3.1.1, the MSD JL Vaccine Bulk inoculated animals show a low-level, i.e. -/+ effect on the thickness of the cerebral cortex seen in 2 specimens and the sub-epithelial layer of the third ventricle, as seen in 1 specimen in Figure 3.9.
Figure 3.9: Grading assessment of the nine additional parameters of animals inoculated with MSD JL

Key:
1  Thickness of the Cerebral Cortex
2  Third Ventricle; Epithelial changes
3  Third Ventricle; Sub-epithelial changes
4  Third Ventricle; Size
5  Lateral Ventricle; Epithelial changes
6  Lateral Ventricle; Sub-epithelial changes
7  Cerebellum; Molecular layer
8  Cerebellum; Purkinje cell layer
9  Cerebellum Granular layer
This differs from the negative control group which graded completely negative. The positive
control inoculated animals graded between Grade One and Grade Two (Figure 3.7). Parameters
2, 4, and 5 were found to be similar to the negative control group, well within the expected
ranges (Figure 3.8 and Appendix I).
Parameter 6 shows four specimens having low-level effects and one specimen having Grade
One level effects. The JL MSD Vaccine Bulk inoculated animals had no effects on the sub-
epithelial layer of the lateral ventricle. Of the cerebellum, JL MSD Vaccine Bulk had a low-level
effect on the molecular layer, 55% or 11 specimens. The negative control for RNVT E also
showed that SKB JL Vaccine Bulk virus had some effects on the molecular layer, as 22.2% or
two specimens had low-level effects and 22.2% or two specimens had Grade One effects. The
JL MSD Vaccine Bulk virus did not show any effects on the other layers of the cerebellum,
whereas the negative control had one specimen showing a low-level -/+ effect on the Purkinje
Cell Layer.
The JL2/4 sub-component virus showed low-level effects on the epithelial layer and size of
the third ventricle, Figure 3.10. The negative control virus in RNVT G showed no effect. The
animals inoculated with the negative control virus displayed evidence of the lateral ventricle
being affected, as did the animals inoculated with the JL2/4 sub-component vaccine virus. The
effect in both cases is low-level, -/+ grade. The negative control virus exhibited 11.1%, or one
animal, showing effects on the molecular layer of the cerebellum, whereas the vaccine virus
showed 33.3%, or three specimens, to be affected. Neither had any effects on the remaining
parameters of the cerebellum.
The thickness of the cerebral cortex of JL5/4 sub-component vaccine virus inoculated animals
exhibited a range of scoring grades. Like the negative control animals it showed a high degree
of negative grading, with few specimens showing Grade One and Grade Two level effects
(Appendix II). Animals inoculated with the positive control virus showed Grade One and Two
level effects. The JL5/4 sub-component virus had a relatively low-level effect on the epithelial
Figure 3.10: Grading assessment of the nine additional parameters of animals inoculated with JL2/4

Key:
1. Thickness of the Cerebral Cortex
2. Third Ventricle; Epithelial changes
3. Third Ventricle; Sub-epithelial changes
4. Third Ventricle; Size
5. Lateral Ventricle; Epithelial changes
6. Lateral Ventricle; Sub-epithelial changes
7. Cerebellum; Molecular layer
8. Cerebellum; Purkinje cell layer
9. Cerebellum Granular layer
Figure 3.11 : Grading assessment of the nine additional parameters of animals inoculated with JL5/4

Key:

1. Thickness of the Cerebral Cortex
2. Third Ventricle; Epithelial changes
3. Third Ventricle; Sub-epithelial changes
4. Third Ventricle; Size
5. Lateral Ventricle; Epithelial changes
6. Lateral Ventricle; Sub-epithelial changes
7. Cerebellum; Molecular layer
8. Cerebellum; Purkinje cell layer
9. Cerebellum Granular layer
layer and size of the third ventricle, as did the JL2/4 sub-component. The JL5/4 component virus produced effects on the lateral ventricle which were more severe than the effects seen with negative control and JL2/4 virus preparations. Like the positive control virus, the JL5/4 variant had Grade One effects on the epithelial layer of the lateral ventricle. Animals inoculated with the positive control scored in the One/Two and Two grades, whereas the animals inoculated with JL5/4 preparation scored mainly in the negative and low-level groupings. The sub-epithelial changes seen in animals inoculated with JL5/4 and positive control preparation were of Grade One and Two levels in some animals.

The effect on the molecular layer of the cerebellum was similar in animals inoculated with the positive control and JL5/4 sub-variant vaccine virus.

3.3.3 Observations of Urabe Mumps Vaccine Inoculated Animals.

This section details the results of the Urabe vaccine and its associated isolates that were analysed in the following three RNVT’s:

- **RNVT B** - SKB Urabe Vaccine Bulk
- **RNVT F** - Lo4/2 Urabe Post-vaccine isolate
- **RNVT D** - Nt5 Urabe Post-vaccine Isolate
Table 3.5: Grading assessment of the Lateral Ventricle size of Urabe vaccine and associated derivatives.

<table>
<thead>
<tr>
<th>Grading</th>
<th>SKB Urabe Vaccine Bulk</th>
<th>Percentage of Controls for RNVT B</th>
<th>Lo4 Urabe Post-Vaccine Isolate.</th>
<th>Percentage of Controls for RNVT F</th>
<th>Nt5 Urabe Post-Vaccine Isolate.</th>
<th>Percentage of Controls for RNVT D</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>21</td>
<td>0</td>
<td>50</td>
<td>30</td>
<td>0</td>
<td>60</td>
</tr>
<tr>
<td>-/+</td>
<td>42.1</td>
<td>10</td>
<td>37.5</td>
<td>60</td>
<td>8.3</td>
<td>40</td>
</tr>
<tr>
<td>+</td>
<td>26.3</td>
<td>30</td>
<td>12.5</td>
<td>10</td>
<td>33.3</td>
<td>0</td>
</tr>
<tr>
<td>+/-</td>
<td>5.3</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>16.7</td>
<td>0</td>
</tr>
<tr>
<td>++</td>
<td>5.3</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>16.7</td>
<td>0</td>
</tr>
<tr>
<td>+++/++++</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+++</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>
The SKB Urabe Vaccine Bulk virus had a range of effects on the size of the lateral ventricle, with 21% of animals scoring negative. The vaccine virus displayed a Grade One level enlarging effect in 26.3% or five specimens Grade One or higher in 5.3% of animals and Grade Two level in another 5.3% of animals (Table 3.5). The pattern of histological scoring for the negative control was in line with the pattern seen in other RNVT's. The positive control virus used in RNVT B produced effects of Grades One to Three (Table 3.5).

The Lo4 Urabe virus displayed Grade One level effects in 10% of animals with the remainder showing no effects or that of a low-level score, essentially identical to the negative control (Table 3.5). The positive control virus showed a majority scoring between Grade One and Grade Two, 91.7%, which is consistent with the pattern of the positive control preparation in other RNVT's (Table 3.3). Animals inoculated with the Nt5 Urabe virus showed a range of grades similar to the animals that were inoculated with the SKB Urabe Vaccine Bulk material. The negative control virus of RNVT D graded similar to RNVT B. The positive control virus of RNVT D showed that the majority of specimens (90%) had a Grade One to Two level effect, which is also consistent with the results of positive control samples examined in other RNVT's. The relatively high scoring profile seen with SKB Urabe Vaccine virus and Nt5 Urabe isolate inoculated animals could possibly be due to the high assay backgrounds as the negative control virus used in RNVT B and D scored slightly higher than its normal pattern seen in other RNVT's (Table 3.3).
Figure 3.12: Grading assessment of the nine additional parameters of animals inoculated with SKB Urabe Vaccine

Key:
1. Thickness of the Cerebral Cortex
10. Third Ventricle; Epithelial changes
11. Third Ventricle; Sub-epithelial changes
12. Third Ventricle; Size
13. Lateral Ventricle; Epithelial changes
14. Lateral Ventricle; Sub-epithelial changes
15. Cerebellum; Molecular layer
16. Cerebellum; Purkinje cell layer
17. Cerebellum Granular layer
In relation to other parameters discussed in section 3.1.1, the SKB Urabe virus inoculated animals had one specimen which showed an enlarging effect on the thickness of the cerebral cortex, the negative control group also had one specimen of a similar nature (Table 3.3 and Figure 3.8). The positive control group animals had a majority of specimens belonging to the Grade One level effects. Both the negative and positive control group animals exhibited no effects on the third ventricle, whereas the SKB Urabe Vaccine virus inoculated animals showed low-level effects on all parameters and Grade One level effects on the epithelial and sub-epithelial layers (Table 3.3, Figures 3.7 and 3.8). Four specimens (21.2%) showed Grade One effects on the epithelial layer and three specimens (15.9%) showed Grade One level effects on the sub-epithelial layer. Both the positive and negative control group animals showed effects on the sub-epithelial layer of the lateral ventricle similar to the levels seen with SKB Urabe vaccine virus inoculated animals. The positive control group animals had more severe effects on the epithelial and sub-epithelial layers than the animals inoculated with the negative control and vaccine viruses (Table 3.3, Figures 3.7 and 3.8). The negative control virus had no effects on the epithelial layer whereas the SKB Urabe Vaccine virus inoculated animals had one specimen showing Grade One/Two level effects, and 5 specimens (26.5%) showing Grade One level effects (Tables 3.3 and 3.5, Appendix I). The negative control virus inoculated animals showed that three specimens, (37.5%) had Grade One level effects. The vaccine virus showed that 3 specimens (16.6%) had Grade One/Two level effects and the same number had Grade One level effects. The SKB Urabe vaccine virus inoculated rats showed effects similar to the animals inoculated with the positive control virus (RNVT B) than the animals inoculated with the negative control virus for variations in the molecular layer of the cerebellum (Table 3.5). The Purkinje cell layer of the SKB Urabe inoculated specimens displayed mainly negative to low-level effects, one specimen had a Grade One level effect. The animals inoculated with the negative control virus gave grades that were negative for all parameters of the cerebellum (Appendix I).
Figure 3.13: Grading assessment of the nine additional parameters of animals inoculated with Lo4 Urabe Sample

Key:
1. Thickness of the Cerebral Cortex
2. Third Ventricle; Epithelial changes
3. Third Ventricle; Sub-epithelial changes
4. Third Ventricle; Size
5. Lateral Ventricle; Epithelial changes
6. Lateral Ventricle; Sub-epithelial changes
7. Cerebellum; Molecular layer
8. Cerebellum; Purkinje cell layer
9. Cerebellum Granular layer
Animals inoculated with the Lo4 Urabe virus showed no effects on the thickness of the cerebral cortex or the third ventricle (Appendix I). The animals inoculated with the negative control virus also had no effects on these parameters. The positive control virus had either negative or low-level effects on these parameters. One animal had low-level effects on the epithelial layer of the lateral ventricle (Appendix I). Specimens derived from the positive control virus inoculated animals showed effects on the epithelial layer of Grade One. Of the animals inoculated with Lo4 virus, one animal exhibited Grade One level effects on the sub-epithelial layer, the same was observed in animals inoculated with the negative control virus (Appendix I). The animals injected with positive control virus had effects of the Grade One and Two levels. There were some low-level effects (16.6%, 2 specimens) in the molecular layer of the cerebellum in Lo4 inoculated animals. The negative control group for this parameter was completely negative while the positive control group had Grade One level effects in some animals. The Purkinje and Granular Cell Layers both remained unaffected in the negative control and Lo4 inoculated Groups (Appendix I).

The thickness of the cerebral cortex showed variation between negative control group animals, and those inoculated with Nt5 in RNVT D (Appendix I). The Nt5 virus inoculated animals had majority of specimens that were negative, one specimen that had low-level effects and two specimens that had Grade One level effects. The Nt5 virus had negative to low-level effects on the third ventricle (Appendix I). The negative control group displayed mainly negative to low-level effects on the epithelial changes of the lateral ventricle whilst two specimens had some measurable effects. The positive control virus showed that the majority of animals had low-level effects on the sub-epithelial part of the lateral ventricle, and only two specimens had Grade One level effects (Appendix I). The negative control group had four specimens that showed low-level effects, whereas the Nt5 inoculated group showed low-level effects on the sub-epithelial layer and two specimens having Grade One level effects. The negative control group
Figure 3.14: Grading assessment of the nine additional parameters of animals inoculated with Nt5 Urabe Sample

Key:
1. Thickness of the Cerebral Cortex
2. Third Ventricle; Epithelial changes
3. Third Ventricle; Sub-epithelial changes
4. Third Ventricle; Size
5. Lateral Ventricle; Epithelial changes
6. Lateral Ventricle; Sub-epithelial changes
7. Cerebellum; Molecular layer
8. Cerebellum; Purkinje cell layer
9. Cerebellum Granular layer
displayed low-level effects on both the molecular and purkinje cell layer of the cerebellum, scoring two specimens in the former and one specimen in the latter category (Appendix I). The animals inoculated with the positive control virus showed low-level to Grade One level effects on the molecular layer but not on the Purkinje cell layer. The Nt5 virus had low-level effects on both layers, five specimens in the former and four specimens in the latter. None of the viruses of the Urabe vaccine virus related group had any effects on the Granular Cell layer (Appendix I).

3.3.4 Observations of Clinical Material Inoculated Animals

This section details the histopathological results of animals that were inoculated with mumps specific unpassaged clinical material, and limitedly passaged, clinical material designated 99-0333 and 99-0333V, respectively.

Animals inoculated with the clinical material, 99-0333, showed variations in size of the ventricle region of the brain compared to animals inoculated with the negative control virus preparation (Table 3.6). One animal (25%) of the 99-0333 group showed Grade one/two effects. The same was observed for the animals that were given Vero cell passaged version of the clinical material 99-0333V. The performance of the negative control in RNVT B was in line with the performances of this material observed in other RNVT’s (Table 3.3). The Grade one level effects seen with the negative control material have also been observed in RNVTs D and I. The distribution of specimens of the positive control virus inoculated animals showed that about 70% specimens showed grade one to two level effects which is also within the acceptable range for LO1/Mu90 strain as seen in other RNVT’s (Tables 3.3 and 3.6).

In relation to other parameters discussed in section 3.1.1, the thickness of the cerebral cortex showed that one specimen (25%) had Grade One level effects (Appendix I). The negative control virus had one specimen, (12.5%) showing low-level effects, whereas the positive control virus had mainly Grade One level effects with a few being extended up to Grade Two.
Table 3.6: Grading assessment of the Lateral Ventricle size of animals inoculated with mumps specific clinical sample and associated isolate.

<table>
<thead>
<tr>
<th>Grading</th>
<th>Clinical Material Sample 99-0333</th>
<th>Clinical Material Sample 99-0333V</th>
<th>Percentage of Controls for RNVT B</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>25</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>-/+</td>
<td>0</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>+</td>
<td>50</td>
<td>75</td>
<td>30</td>
</tr>
<tr>
<td>+/++</td>
<td>25</td>
<td>25</td>
<td>20</td>
</tr>
<tr>
<td>++</td>
<td>0</td>
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</tr>
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<td>0</td>
<td>10</td>
</tr>
<tr>
<td>+++</td>
<td>0</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>


Figure 3.15: Grading assessment of the nine additional parameters of animals inoculated with Clinical Material 99-0333

Key:
1. Thickness of the Cerebral Cortex
2. Third Ventricle; Epithelial changes
3. Third Ventricle; Sub-epithelial changes
4. Third Ventricle; Size
5. Lateral Ventricle; Epithelial changes
6. Lateral Ventricle; Sub-epithelial changes
7. Cerebellum; Molecular layer
8. Cerebellum; Purkinje cell layer
9. Cerebellum Granular layer
The 99-0333 clinical material and positive and negative control viruses had no detectable effects on any other parameters of the third ventricle (Appendix I). The 99-0333 material had more pronounced effects on the parameters of the lateral ventricle than the corresponding negative control virus. Two animals (50%) inoculated with 99-0333 material displayed Grade One level effects and one animal (25%) had Grade One/Two level effects. The positive control virus showed Grade One level effects on the epithelial layer (Appendix I). The sub-epithelial layer of the lateral ventricle in two specimens (50%) was effected by a Grade One and in one specimen (25%) by a Grade Two.
Figure 3.16: Grading assessment of the nine additional parameters of animals inoculated with Clinical Material 99-0333V

Key:
1. Thickness of the Cerebral Cortex
2. Third Ventricle; Epithelial changes
3. Third Ventricle; Sub-epithelial changes
4. Third Ventricle; Size
5. Lateral Ventricle; Epithelial changes
6. Lateral Ventricle; Sub-epithelial changes
7. Cerebellum; Molecular layer
8. Cerebellum; Purkinje cell layer
9. Cerebellum Granular layer
The animals receiving negative control virus had low-level effects on the sub-epithelial layer whilst those receiving the positive control had Grade One and Grade Two level effects (Appendix I). The 99-0333 virus showed to be negative with a degree of low-level effect on the molecular layer of the cerebellum as did the negative control virus group. The positive control group mostly had Grade One level effects on the molecular layer and Purkinje Cell layer whilst one specimen had Grade Two level effects on the Granular Layer (Appendix I).

Both 99-0333 material and negative control virus showed no effects on the Granular Layer. The thickness of the cerebral cortex of animals inoculated with 99-0333V and negative control virus was not effected at all. Unlike the negative and positive control viruses, 99-0333V isolate had an effect on the third ventricle (Appendix I). One specimen (25%) had a Grade One level effects on the epithelial layer. One sample (25%) had low-level effects on the sub-epithelial layer and had no effects on the size of the third ventricle. The 99-0333V inoculated animals had 3 specimens (75%) which showed Grade One level effects on the size of the lateral ventricle (Appendix I). One specimen (25%) had Grade One/Two level effects on the epithelial layer. Three specimens (75%) had Grade One level effects and one specimen (25%) had Grade Two level effects on the sub-epithelial layer. The negative control group had one specimen (25%) having Grade One level effects on the sub-epithelial layer. The 99-0333V isolate had one specimen (25%) showing Grade One level effects on the molecular layer (Appendix I). The other two parameters of the cerebellum region of the animals inoculated with 99-0333V isolate did not show any variations.
3.3.5 Observations of Diluent Sample Inoculated Animals.

The animals discussed in this section were inoculated with the medium used to dilute the virus stock samples. Technically this is another version of the negative control material but for ethical reasons was not chosen for that purpose in this study. For the composition of the diluent sample see Section 2.1.

Animals inoculated with the diluent material did not show many changes in the ventricle region of the brain (Table 3.7). About the same number of animals also did not show any changes in the negative control group. However, in the diluent group one animal (5.3%) showed changes that is classified as Grade One (Figure 3.17). This could be due to an error in the slide reading or of the tissue processing procedure that may have caused tissue damage.

All of the additional parameters assessed were negative with the exception of the sub-epithelial layer of the lateral ventricle, that showed one specimen having low-level effects (Appendix I).

The negative control virus group was also negative for all additional parameters except for the epithelial layer, sub-epithelial layer and size of the lateral ventricle (Appendix I). The positive control virus group displayed a mixture of negative, low-levels to positively detectable effects on all additional parameters as seen with the inoculated animals in other RNVTs in this study.
Table 3.7: Grading Assessment of the Lateral Ventricle size of animals inoculated with the diluent sample.

<table>
<thead>
<tr>
<th>Grading</th>
<th>Diluent Samples</th>
<th>Percentage of Controls for RNVT J</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>89.5</td>
</tr>
<tr>
<td>-/+</td>
<td>5.3</td>
<td>12.5</td>
</tr>
<tr>
<td>+</td>
<td>5.3</td>
<td>25</td>
</tr>
<tr>
<td>+/++</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>++</td>
<td>0</td>
<td>37.5</td>
</tr>
<tr>
<td>++/++++</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+++</td>
<td>0</td>
<td>25</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>
Figure 3.17: The Diluent Sample showing a Grade One effect
Figure 3.18: Grading assessment of the nine additional parameters of animals inoculated with the Diluent

Key:
1. Thickness of the Cerebral Cortex
2. Third Ventricle; Epithelial changes
3. Third Ventricle; Sub-epithelial changes
4. Third Ventricle; Size
5. Lateral Ventricle; Epithelial changes
6. Lateral Ventricle; Sub-epithelial changes
7. Cerebellum; Molecular layer
8. Cerebellum; Purkinje cell layer
9. Cerebellum Granular layer
3.4 Histopathological data of samples obtained from FDA.

The table below shows a summary of data generated for the lateral ventricle size of brain samples of animals inoculated with five different samples received from FDA, USA. All samples were supplied and tested coded. The data presented in this section, therefore, could not be attributed to any particular strain of virus, mumps or otherwise, until the decoding is performed by FDA.

In this part, histopathological data of five different strains of mumps viruses, evaluated in five different tests are presented. The tests were conducted in the following formats:

- RNVT F - FDA sample A
- RNVT I - FDA sample B
- RNVT D - FDA sample C
- RNVT E - FDA sample D
- RNVT H - FDA sample E

Each RNVT had an appropriate positive control virus and a negative control virus, identical to the assay format applied for NIBSC mumps virus strains testing. The data shown in Table 3.8 indicated that 31.6% animals inoculated with sample A did not present many effects on the lateral ventricle size. Sixty-three percent of animals showed low-level effects on the lateral ventricle size, whilst one animal (5.3%) had effects of the grade level One/Two (Table 3.8). In contrast, the animals inoculated with sample B had more pronounced effects on the lateral ventricle sizes as all of the animals showed measurable effects ranging from Grade One to Grade Four.
Table 3.8: Summary of data of the lateral ventricle size of animals inoculated with FDA samples.

<table>
<thead>
<tr>
<th>Grading</th>
<th>FDA A</th>
<th>Controls for RNVT F</th>
<th>FDA B</th>
<th>Controls for RNVT I</th>
<th>FDA C</th>
<th>Controls for RNVT D</th>
<th>FDA D</th>
<th>Control for RNVT E</th>
<th>FDA E</th>
<th>Controls for RNVT H</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>-</td>
<td>31.6</td>
<td>0 60</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>54.5</td>
<td>35</td>
<td>0 40</td>
<td>55</td>
<td>0 54.5</td>
</tr>
<tr>
<td>+/-</td>
<td>63.1</td>
<td>8.3 40</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>27.2</td>
<td>33.3</td>
<td>10 36.3</td>
<td>15</td>
<td>0 60</td>
</tr>
<tr>
<td>+</td>
<td>0</td>
<td>33.3 0</td>
<td>13.6</td>
<td>45.5</td>
<td>18.2</td>
<td>47.6</td>
<td>60</td>
<td>9.1</td>
<td>25</td>
<td>0 10</td>
</tr>
<tr>
<td>+/-++</td>
<td>5.3</td>
<td>16.7 0</td>
<td>0</td>
<td>18.1</td>
<td>0</td>
<td>14.3</td>
<td>0</td>
<td>5</td>
<td>10</td>
<td>0 11.1</td>
</tr>
<tr>
<td>++</td>
<td>0</td>
<td>41.7 0</td>
<td>40.9</td>
<td>27.2</td>
<td>9.1</td>
<td>4.8</td>
<td>20</td>
<td>0</td>
<td>20</td>
<td>10 5</td>
</tr>
<tr>
<td>++/++++</td>
<td>0</td>
<td>0 0</td>
<td>0</td>
<td>9.1</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>0 10</td>
<td>0</td>
<td>0 0 0</td>
</tr>
<tr>
<td>+++</td>
<td>0</td>
<td>0 0</td>
<td>36.4</td>
<td>0 0</td>
<td>0</td>
<td>0</td>
<td>40</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+++/++++</td>
<td>0</td>
<td>0 0</td>
<td>4.5</td>
<td>0 0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0 0</td>
<td>0</td>
<td>0 0 0</td>
</tr>
<tr>
<td>++++</td>
<td>0</td>
<td>0 0</td>
<td>4.5</td>
<td>0 0</td>
<td>0</td>
<td>0</td>
<td>30</td>
<td>0</td>
<td>0</td>
<td>0 0 0</td>
</tr>
<tr>
<td>++</td>
<td>0</td>
<td>0 0</td>
<td>4.5</td>
<td>0 0</td>
<td>0</td>
<td>0</td>
<td>30</td>
<td>0</td>
<td>0</td>
<td>0 0 0</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100 100</td>
<td>100</td>
<td>100 100</td>
<td>100</td>
<td>100 100</td>
<td>100</td>
<td>100 100</td>
<td>100</td>
<td>100 100</td>
</tr>
</tbody>
</table>

114
Sixty seven percent animals inoculated with FDA sample C had effects on the lateral ventricle size ranging from Grade One to Grade Two (Table 3.8). Thirty three percent of specimens could not be conclusively assigned into the negative group and therefore were given -/+ scoring. Thirty five percent of animals inoculated with FDA sample D were completely negative for any changes in the size of lateral ventricle (Table 3.8). Fifteen percent of animals could not be scored definitively and therefore were placed in the -/+ group. The remaining 50% of animals had variations in the size of lateral ventricle that ranged from Grade One to Grade Two. Finally, the animals inoculated with the FDA sample E did not show any changes in lateral ventricle size (Table 3.8).
Figure 3.19: Grading assessment of the nine additional parameters of animals inoculated with the FDA A Strain

Key: 1 Thickness of the Cerebral Cortex

2 Third Ventricle; Epithelial changes

3 Third Ventricle; Sub-epithelial changes

4 Third Ventricle; Size

5 Lateral Ventricle; Epithelial changes

6 Lateral Ventricle; Sub-epithelial changes

7 Cerebellum; Molecular layer

8 Cerebellum; Purkinje cell layer

9 Cerebellum Granular layer
Figure 3.20: Grading assessment of the nine additional parameters of animals inoculated with the FDA B Strain

Key:

1. Thickness of the Cerebral Cortex
2. Third Ventricle; Epithelial changes
3. Third Ventricle; Sub-epithelial changes
4. Third Ventricle; Size
5. Lateral Ventricle; Epithelial changes
6. Lateral Ventricle; Sub-epithelial changes
7. Cerebellum; Molecular layer
8. Cerebellum; Purkinje cell layer
9. Cerebellum Granular layer
It is noticeable from data shown in Table 3.8 that all animals inoculated with the negative control virus preparation either showed no effects or had some low level effects up to Grade One. However, this was not seen in the RNVT I, where the animals inoculated with the negative control virus preparation had 28% of specimens showing Grade One or higher effects. This may attribute to the higher neurovirulence profile of FDA sample B.

In relation to the other parameters examined in this study, it was observed that the FDA sample A had low level effects (-/+) on the epithelial and sub-epithelial layers of the third ventricle as well as on the molecular layer of the cerebellum (Appendix I). The FDA sample B also had some low-level effects on the thickness of the cerebral cortex, the epithelial layer of the third ventricle and on the sub-epithelial layer (Appendix I). Animals inoculated with this strain also showed effects ranging from Grade One to Two for the size of the third ventricle and on the epithelial and sub-epithelial layers of the lateral ventricle. The FDA sample C had some effects in a minority of inoculated animals in the regions of the cerebral cortex, molecular layer of the cerebellum and the epithelial layer of lateral ventricle. One specimen which displayed a low-level effect in the molecular layer of cerebellum also had a very low level effect on the Purkinjie cell layer but no effect on the Granular layer (Appendix I)

The FDA sample D did not show any effects in the inoculated animals on the size of the third ventricle but displayed low-level effects on the epithelial and sub-epithelial layers (Appendix I). One specimen had a Grade One level effect on the size of third ventricle but this was not remarkable, as all other animals of this group did not show any effects on the third ventricle. Thirty eight percent of animals inoculated with FDA sample D also had a Grade One level effect on the molecular layer, 5% on the Purkinjie cell layer and another 5% on the Granular layer (Appendix I).
Figure 3.21: Grading assessment of the nine additional parameters of animals inoculated with the FDA C Strain

Key:
1. Thickness of the Cerebral Cortex
2. Third Ventricle; Epithelial changes
3. Third Ventricle; Sub-epithelial changes
4. Third Ventricle; Size
5. Lateral Ventricle; Epithelial changes
6. Lateral Ventricle; Sub-epithelial changes
7. Cerebellum; Molecular layer
8. Cerebellum; Purkinje cell layer
9. Cerebellum Granular layer
Figure 3.22: Grading assessment of the nine additional parameters of animals inoculated with the FDA D Strain

Key:
1. Thickness of the Cerebral Cortex
2. Third Ventricle; Epithelial changes
3. Third Ventricle; Sub-epithelial changes
4. Third Ventricle; Size
5. Lateral Ventricle; Epithelial changes
6. Lateral Ventricle; Sub-epithelial changes
7. Cerebellum; Molecular layer
8. Cerebellum; Purkinje cell layer
9. Cerebellum Granular layer
Figure 3.23: Grading assessment of the nine additional parameters of animals inoculated with the FDA E Strain

Key: 1 Thickness of the Cerebral Cortex
    2 Third Ventricle; Epithelial changes
    3 Third Ventricle; Sub-epithelial changes
    4 Third Ventricle; Size
    5 Lateral Ventricle; Epithelial changes
    6 Lateral Ventricle; Sub-epithelial changes
    7 Cerebellum; Molecular layer
    8 Cerebellum; Purkinje cell layer
    9 Cerebellum Granular layer
Interestingly, the FDA sample E did not show any recordable effects on the lateral ventricle size of the brains of inoculated animals, but did produce some effects, Grade One, in one animal on the thickness of cerebral cortex (Appendix I). The negative control virus inoculated animals also gave similar scorings. A few animals inoculated with FDA E sample also showed low-level changes in the epithelial layer of lateral ventricle and Purkinje cell layer.

3.5 FDA Scoring Procedure

The scoring of histopathological slides for lateral ventricle enlargement, carried out at FDA, USA, was different to the method applied during this study. At NIBSC, the scoring was based on a visual observation, under microscope, where any enlargement in the size of the lateral ventricle region was assigned from being completely negative (-) to Grade Four (++++), in line with the scoring procedure developed for the monkey model (Afzal et al 1999a).

In contrast, the FDA procedure was based on recording a numerical value for the lateral ventricle region. The numerical value was the percentage area of the total brain that the lateral ventricle occupied, excluding the brain stem. This method gave an estimation of the degree of hydrocephalus caused in the brain.

Both the FDA and NIBSC samples were read using the in-house method as well as the method developed by FDA. Both sets of data are reported in Appendices I and III.

3.5.1 Calculation of mean values and their interpretation

In order to calculate the mean values of lateral ventricle areas, percentage figures obtained after each numerical recording were added together and then divided by the total number of samples examined in that group.
Table 3.9: The lateral ventricle mean value using the FDA Scoring method.

<table>
<thead>
<tr>
<th>Virus Group</th>
<th>Group Mean Percentage of Lateral Ventricle Enlargement.</th>
</tr>
</thead>
<tbody>
<tr>
<td>FDA B Sample</td>
<td>24.04%</td>
</tr>
<tr>
<td>Lo1/Mu90</td>
<td>12.04%</td>
</tr>
<tr>
<td>FDA C Sample</td>
<td>5.71%</td>
</tr>
<tr>
<td>FDA D Sample</td>
<td>5.2%</td>
</tr>
<tr>
<td>JL5/4</td>
<td>4.8%</td>
</tr>
<tr>
<td>Ntl5 Urabe Sample</td>
<td>4.4%</td>
</tr>
<tr>
<td>Clinical Material 99-0333/1V</td>
<td>3.5%</td>
</tr>
<tr>
<td>Clinical Material 99-0333</td>
<td>2.8%</td>
</tr>
<tr>
<td>Urabe Vaccine Bulk (SKB)</td>
<td>2.7%</td>
</tr>
<tr>
<td>FDA E Sample</td>
<td>2.58%</td>
</tr>
<tr>
<td>Lo4/2 Urabe Sample</td>
<td>2.0%</td>
</tr>
<tr>
<td>FDA A Sample</td>
<td>1.3%</td>
</tr>
<tr>
<td>JL2/4</td>
<td>1.0%</td>
</tr>
<tr>
<td>JL Vaccine Bulk (SKB)</td>
<td>0.42%</td>
</tr>
<tr>
<td>Diluent Only</td>
<td>0.28%</td>
</tr>
<tr>
<td>JL Vaccine Bulk (MSD)</td>
<td>0.14%</td>
</tr>
</tbody>
</table>
The percentage mean values of the lateral ventricle area of the brain in each group of inoculated animals may be useful in predicting levels of neurovirulence of each mumps virus strain in neonatal rats.

The FDA B sample gave the highest mean percentage of all the samples tested, it showed to be twice as neurovirulent than the positive control Lo1/Mu90 (Table 3.9). The JL MSD vaccine bulk had a mean percentage value of 0.14%, which was the lowest mean scoring. Interestingly, the diluent sample showed a group mean value of 0.28%. Although this score is higher than JL MSD vaccine bulk it is due to an anomalous sample (Figure 3.9, Table 3.7). The JL SKB vaccine bulk preparation that was routinely used as an assay negative control in this study had a percentage mean value of 0.42% (Table 3.9). As seen with the monkey neurovirulence studies, the JL vaccine based samples had less severe effects on the size of lateral ventricle in rats than the Urabe vaccine related samples.

The percentage mean values reported in Table 3.9 could be useful markers in establishing a correlation between various strains and their associated neurovirulent potential in neonatal rats. However, as seen with the data of the diluent inoculated group, false results could easily contribute to the group average that may not necessarily be correct.

3.5.2 Unpaired Students T-test

The unpaired students T-test was applied to observe statistical significance between data sets in terms of the probability (P) value. The further away the P value is from 1.0, the less probability there is to suggest a statistical relationship between the two data sets. A relationship is considered to be relevant if the P value is above 0.01, the value represents a universal cut-off point. Conversely, two data sets are considered to be significantly different if the P value is less than 0.01 with increasing significance being attached to the difference with decreasing P values, below 0.01.

Using this test, it was possible to distinguish between wild-type and vaccine strains of mumps in terms of their neuropathological performances in the neo-natal rat model.
<table>
<thead>
<tr>
<th>LO4/2 Urabe</th>
<th>NT5 Urabe</th>
<th>SKB JL Bulk</th>
<th>MSD Bulk</th>
<th>JL2/4</th>
<th>JL5/4</th>
<th>Clinical Material</th>
<th>Clinical Material 1V</th>
<th>Diluent Only</th>
<th>Lo1/Mu90</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.258</td>
<td>0.149</td>
<td>0.935</td>
<td>4.71x10^{-6}</td>
<td>0.0034</td>
<td>0.0838</td>
<td>0.908</td>
<td>0.5099</td>
<td>6.14x10^{-5}</td>
<td>2.99x10^{-6}</td>
</tr>
<tr>
<td>-</td>
<td>0.036</td>
<td>0.439</td>
<td>2.65x10^{-5}</td>
<td>0.041</td>
<td>0.018</td>
<td>0.38</td>
<td>0.155</td>
<td>4.02x10^{-4}</td>
<td>3.4x10^{-7}</td>
</tr>
<tr>
<td>-</td>
<td>0.21</td>
<td>1.64x10^{4}</td>
<td>1.87x10^{-3}</td>
<td>0.779</td>
<td>0.528</td>
<td>0.722</td>
<td>5.82x10^{-4}</td>
<td>7.28x10^{-5}</td>
<td>Nt5 Urabe</td>
</tr>
<tr>
<td>-</td>
<td>6.37x10^{-3}</td>
<td>0.063</td>
<td>0.122</td>
<td>0.984</td>
<td>0.748</td>
<td>0.015</td>
<td>7.9x10^{-7}</td>
<td>SKB JL Bulk</td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>6.72x10^{-3}</td>
<td>6.799x10^{-5}</td>
<td>1.26x10^{-6}</td>
<td>1.14x10^{-5}</td>
<td>0.499</td>
<td>1.14x10^{-9}</td>
<td>MSD JL Bulk</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>7.6x10^{-4}</td>
<td>0.025</td>
<td>7.989x10^{-3}</td>
<td>0.046</td>
<td>4.21x10^{-9}</td>
<td>JL2/4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>0.443</td>
<td>0.618</td>
<td>2.6x10^{-4}</td>
<td>1.2x10^{-4}</td>
<td>JL5/4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>0.6835</td>
<td>1.9x10^{-4}</td>
<td>0.023</td>
<td>Clinical Material</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>2.5x10^{-4}</td>
<td>0.035</td>
<td>Clinical Material 1V</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>9.354x10^{-9}</td>
<td></td>
<td>Diluent only</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td></td>
<td></td>
<td>LO1/Mu90</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3.10: The P value of Virus samples tested at NIBSC.
Animals inoculated with Lo1/Mu90 did not show any relationship, in terms of P values, with any other strain tested. In this analysis, the Lo1 strain closely resembled the clinical material 99-0333 (P 0.023) and its cell culture harvest 99-0333/1V (P 0.035). But the significance of this relationship can not be conclusively predicated as the dose of the clinical material and its cell culture harvest was not critically defined. The positive control virus, Lo1.Mu90, was also statistically different than the negative control (P 7.9x10^-7).

In terms of neuropathological effects the negative control virus, SKB JL vaccine bulk, showed relationships with unpassaged clinical material sample 99-0333 (P 0.748) and the culture harvest of the clinical material 99-0333/1V (P 0.984), JL5/4 (P 0.122), JL2/4 (P 0.063) than the other strains.

In terms of the P value, a stronger relationship was observed between the diluent sample and MSD JL vaccine bulk preparation (P 0.499), both of which showed very little, or no, effects on the lateral ventricle size enlargement in the inoculated rats.

Interestingly, the MSD JL vaccine bulk did not show any neuropathological resemblance with its two sub-component viruses, JL5/4 (P 6.799x10^-5) and JL2/4 (P 6.72x10^-3); and was statistically different from them. The sub-components were given four passages in Vero cells.

The SKB Urabe vaccine bulk show significant neuropathological resemblance with the clinical material 99-0333 (P 0.908) and its cell culture harvest 99-0333/1V (P 0.838), SKB JL vaccine bulk (P 0/935), Nt5 (P 0.149) and Lo4 (P 0.258) isolates. This indicates that strains with minor differences in their neurovirulent properties would be difficult to distinguish with this test.
Table 3.11: The P values of viruses obtained from the FDA.

<table>
<thead>
<tr>
<th></th>
<th>FDA A</th>
<th>FDA B</th>
<th>FDA C</th>
<th>FDA D</th>
<th>FDA E</th>
</tr>
</thead>
<tbody>
<tr>
<td>FDA A</td>
<td>-</td>
<td>7.107\times10^{-12}</td>
<td>6.9\times10^{-5}</td>
<td>0.004</td>
<td>0.242</td>
</tr>
<tr>
<td>FDA B</td>
<td>7.107\times10^{-12}</td>
<td>-</td>
<td>1.26\times10^{-9}</td>
<td>1.08\times10^{-8}</td>
<td>3.44\times10^{-11}</td>
</tr>
<tr>
<td>FDA C</td>
<td>6.9\times10^{-5}</td>
<td>1.26\times10^{-9}</td>
<td>-</td>
<td>0.972</td>
<td>0.0791</td>
</tr>
<tr>
<td>FDA D</td>
<td>0.004</td>
<td>1.08\times10^{-8}</td>
<td>0.972</td>
<td>-</td>
<td>0.058</td>
</tr>
<tr>
<td>FDA E</td>
<td>0.242</td>
<td>3.44\times10^{-11}</td>
<td>0.0791</td>
<td>0.0791</td>
<td>-</td>
</tr>
</tbody>
</table>
Given the fact that the identity of the FDA samples are not in at this time it is difficult to surmise if the differences seen in the p values in Table 3.11 would bear any descriptive values. However, the data presented above indicates that the FDA A sample is significantly more related to samples D and E than samples B and C. However, FDA B sample is unique in its neuropathological characteristic, similar to the NIBSC Lo1/Mu90 strain, and does not resemble any other strains tested in the panel. In terms of p values the sample C is more closer to samples D and E than samples A and B. It remains to be seen whether the clinical and isolation details of these strains also support the association established on the neuropathological profile observed in this study.
**Discussion**

Mumps is a common childhood illness which is characterised by swelling of the parotid glands. Its viral aetiological agent was originally identified by Johnson and Goodpasture in 1935. Mumps virus is transmitted by droplet spread and infects the nasal and upper respiratory mucosal epithelium. Dissemination of the virus leads to infection of the visceral organs and the central nervous system. This can cause a variety of mumps-associated complications including: epididymo-orchitis, pancreatitis, meningo-encephalitis and spontaneous abortion (see Section 1.1). Prophylactic vaccines have led to a reduction in the number of cases of mumps illness and its associated clinical complications.

It has been observed that some brands of vaccines, notably those containing the Urabe, L-3 and LZ strains, are associated with significant adverse effects in vaccine recipients. The complications range from minor allergic reactions to meningitis and encephalitis. These vaccines were tested by the manufacturers using the monkey neurovirulence test during the stages of vaccine development and evaluation. However, it is understood the test had failed to detect, or predict the lack of, virulence that may be associated with these vaccines for human recipients (Buynak and Hilleman, 1966). Therefore, it is vital that alternative animal models, in addition to the monkey model, should be explored on the assumption that one may prove to be a better substrate for the prediction of vaccine associated neurovirulence.

The European Pharmacopoeia stipulates that all live viral vaccines are to be tested in monkeys for side-effects, including neurovirulent activity, before being licensed for public use. While the monkey model works satisfactorily to test polio and yellow fever vaccines it has not been a very accurate predictor of the neurovirulent potential of mumps vaccines. The current monkey model requires the inoculation of mumps vaccine preparation directly into the brain of ten animals which, after an incubation period of three to four weeks, are examined histopathologically for any signs of virus specific lesions in the brain.

A Japanese group has recently investigated the marmoset model for mumps neurovirulence.
testing and preliminary results are appealing (Saika et al 2002). However, a model based on small laboratory animals for mumps vaccine neurovirulence testing is highly desirable for several reasons including the ethics, cost, ease of maintaining and handling animals and other technical grounds under which large number of animals could be used to generate statistically meaningful data.

In the current study, Lewis rats were inoculated with mumps virus vaccines, vaccine associated isolates and wild type virus some of which had been tested in the monkey model previously at NIBSC or elsewhere (Rubin et al 1998a, 1998b, 2000). The key objective of this study was to evaluate the rat model for its susceptibility to various mumps virus vaccine strains and to identify whether the test could discriminate between closely related vaccine preparations which may or may not be suitable for immunisation.

4.1 Lateral ventricle size variation - a key parameter

It has been shown by Rubin et al (1998a, 1998b; 2000) that the lateral ventricle of the neonatal rat brain is the most susceptible area showing histopathological alterations following I.C. inoculation of mumps virus strains. It is suggested that the change in the size of the lateral ventricle is induced by an alteration in the equilibrium flow of the cerebrospinal fluid. The virus is thought to infect the cells surrounding the ventricle which control this process, resulting in an increase in the flow of CSF into the ventricular space and distending the lateral ventricle. This causes a pathological condition known as hydrocephalus. This project is aimed to examine the degree to which a particular mumps virus strain distends the lateral ventricle over a given study period, which is speculated to be linked to the neuroinvasive properties of the strain in developing rat brains.

4.2 The Jeryl Lynn vaccine group.

The JL vaccine strain specific materials used in this study were derived from the following different sources:

- JL-MSD vaccine bulk
JL-SKB vaccine bulk

JL2/4 isolate from JL-MSD vaccine bulk

JL5/4 isolate from JL-MSD vaccine bulk

The difference between the two vaccine bulks is based on the residual virus components, which in the case of JL-MSD has both the JL-2 and JL-5 sub-variants. In contrast, JL-SKB vaccine is derived from the JL-5 sub-variant. Both vaccines have been licensed in the UK and several other countries. None of the JL based vaccines has been associated with the induction of any serious adverse effects in vaccinated children. Based on the proven clinical safety of these vaccines, and considering the residual component purity of the vaccine(s), it was considered that JL SKB vaccine bulk preparation would be suitable to be used as a negative control material in this study. Histopathological data of the lateral ventricle of brains of animals inoculated with various versions of the JL strain revealed that between the two commercial vaccines, the JL SKB material was slightly more reactive than JL MSD vaccine bulk. While the JL MSD vaccine preparation did not produce any effects in any inoculated animals, the JL SKB vaccine preparation had Grade One level effects in 3.4% of animals and very low levels effects on another 40% of inoculated animals (Table 3.3). These findings are further supported by data presented in Table 3.10 which indicate that the mean group percentage for the lateral ventricle size was 0.14% for JL MSD vaccine and 0.42% for JL SKB vaccine. This may be wholly attributed to the component variability of the two vaccines or the JL5 component of the JL SKB vaccine bulk may have gained increased neurovirulence during its passaging in cell culture after isolation from MSD vaccine.

Human recipients of the JL SKB vaccine show no significant adverse events and so the low level of neurovirulence associated with it in this study may not have any cause for concern.

Additionally, between the two component viruses of JL vaccine, JL2/4 and JL5/4, that were passaged 4 times in Vero cells, the JL-5/4 was shown to be more neurovirulent than JL2/4 (Tables 3.4 and 3.9). This adds to the earlier speculation made above that the JL5 sub-variant may gain some virulence during cell culture passaging. It is also noteworthy that JL5/4 was
passaged in Vero cells while the JL5 clone originally used by SKB to generate the vaccine seed was isolated and passaged in CEF cells.

All JL strain associated viruses were examined in two tests, RNVT E and RNVT G, which showed a slight discrepancy between them in terms of the overall performance of positive control virus samples. While in RNVT E the positive control sample produced more than Grade One level effects in nearly 100% of animals this was not seen in RNVT G where similar effects were observed in only 60% of animals. This could be due to an experiment to experiment variation, though overall in this study the experimental consistency was reproducible (see Table 3.3). It was thought that further passages of vaccine strains in vitro would lead towards further attenuation of the virus; however, data presented in this study does not support this notion. It is possible that the vaccine virus may generate a progeny of sub-variants during in vitro culturing that contribute to the enhancement of neurovirulence in comparison to the original vaccine bulk. It would be interesting to characterise the cell culture harvests of JL2/4 and JL5/4 to identify the existence of the quasi-species present.

The results of neurovirulence testing of JL based vaccines could be compared to their characteristics in the field. In the RNVT’s, JL vaccine inoculated animals showed little or no neurological effects which confirms the clinical safety of these vaccines. The rat model has also shown the possibility that further passaging of JL viruses in Vero cells increases the neurovirulent potential of the virus.

The data presented conclude that the neo-natal rat model is able to distinguish between the vaccine strain and some of its derivatives.

4.3 The Urabe vaccine group.

There were three different preparations related to Urabe vaccine virus which were evaluated in this study. They are described below and in Chapter 2.

SKB -Urabe vaccine bulk

Lo4/2, a post-vaccine isolate from an asymptomatic individual, grown in Vero cells
Nt5, a post-vaccine isolate from a symptomatic individual grown in Vero cells. Urabe mumps vaccine has been implicated in the development of aseptic meningitis and other complications in vaccine recipients. Due to these reasons the vaccine was withdrawn from the immunisation schedule in the UK in 1992.

The Urabe vaccine was produced from a plaque picked clone and therefore it was thought that the finished vaccine preparation would contain no residual component viruses. However, nucleotide sequence analyses of the Urabe vaccine strain and of isolates derived from healthy or diseased individuals following vaccination with Urabe revealed the existence of an additional population of viruses in the vaccine, which had accumulated point mutations in various regions of the genome (Afzal et al 1994, Yates et al 1996, Brown et al 1996). Examination of the HN region of the additional viruses has identified a mutation at amino acid 335 which may play a role in both defining the antigenicity of the virus as well as in inducing a neutralising antibody response in vaccine recipients. It has been suggested that amino acid 335 of the HN protein maybe associated with the virulence and attenuation of Urabe mumps vaccine strain (Brown et al 1996, Afzal et al 1998). All Urabe vaccine associated isolates that were originally derived from vaccinees with a range of post-vaccination adverse effects, ranging from swollen glands to meningitis, have had a lysine amino acid residue at position 335 of the HN protein gene sequence, while isolates derived from healthy individuals, who did not show any symptoms following Urabe mumps vaccination had a glutamic amino acid residue at this position. It is notable that the Lo-4 isolate had a glutamic acid residue, and Nt5 had a lysine residue at position 335 of the HN protein (Afzal et al 1998).

The data regarding the lateral ventricle size of the Urabe group presented in Tables 3.5 and 3.9 show that amongst the viruses tested, the animals inoculated with SKB-Urabe vaccine bulk (2.7%) and Nt5 (4.4%) were more severely affected than the animals inoculated with Lo4 (2%). In the Lo4 group, only 10% of animals showed effects that could be graded to level one, while both in SKB-Urabe vaccine bulk and Nt5 inoculated animals nearly 40% of animals showed effects that were graded from levels One to Two. It is notable that the negative control...
virus in RNVTs B and D, in which SKB-Urabe vaccine bulk and Nt5 preparations were examined respectively, showed a slightly higher background scoring than seen in RNVT F, where the Lo4 was tested. This discrepancy, if taken into account, could only lead to a reduction of 10% of animals currently classified in higher groups of SKB-Urabe vaccine bulk and Nt5 inoculated animals. The fact that both SKB-Urabe vaccine bulk and Lo4 presented almost the same degree of neurovirulence scoring pattern in neonatal rats is interesting and could lead one to speculate that the SKB-Urabe vaccine bulk may be mostly composed of the Lo4 type sub-component. Alternatively, it is also possible to suggest that the Nt5 type component present in the Urabe vaccine bulk is slower to replicate in rat brains than the Nt5 type component grown in tissue culture. The composition, in terms of residual sub-variant in SKB-Urabe vaccine bulk used should be determined in future investigations.

Further analysis of Table 3.9 shows that the degree of enlargement of the lateral ventricle size was much more severe in animals inoculated with Nt5 than animals inoculated with unpassaged vaccine bulk. This confirms the notion that passages of mumps virus in Vero cells may contribute towards the enhancement of neurovirulence for the rat brain.

4.4 The Clinical Material Group

All previous studies related to the neurovirulence testing of mumps virus have been carried out with vaccine preparations or wild type strain preparations grown in cell culture. The assessment of unpassaged mumps specific clinical material for neurovirulence, in line with other culture isolates and vaccine preparations, has never been performed in any animal model. In this respect the current study is novel and unique. The clinical material designated, 99-0333, was originally derived in 1999 from a child with parotitis and was supplied to NIBSC by the Health Protection Agency, Colindale, London. This material was passaged once in Vero cells to produce the second generation material designated as 99-0333V. Neurovirulence data of lateral ventricle size enlargement reported in Table 3.6 showed that 75% of animals inoculated with 99-0333 material had Grade One level or higher effects. This is in contrast to 99-0333V inoculated
animals where 100% of animals showed Grade One or higher level effects leading to the suggestion that the cell culture harvest was possibly more virulent than the original clinical material. However it is important to report that the clinical sample, as well as its associated cell culture derived harvest, was inoculated into only four animals. Additionally, both preparations were not titrated to determine their virus titres; therefore, the two group of animals may have received unequal amounts of infectious virus and the observed discrepancies could be due to the dosage variability. A larger number of animals should be inoculated to obtain statistically meaningful results. Additionally, based on the scoring profile of the negative control virus inoculated animals in RNVT B, it is reasonable to suggest that some animals may have been artificially scored higher due to the overall higher test background readings.

4.5 The Diluent Group

The diluent comprised MEM that was used to dilute stock virus preparations prior to the inoculation of animals. Although MEM could have been used as the main negative control sample in the RNVTs conducted, the SKB JL vaccine bulk preparation was used as the principle negative control sample throughout this study. This was for historical reasons as the JL SKB based mumps vaccine has not been shown to be associated with any neurovirulence in vaccine recipients. The use of the diluent as a negative control sample in each assay could have been viewed as an unnecessary waste of animals.

It is interesting to observe that data shown in Table 3.7 reveal that nearly 90% of animals inoculated with the diluent sample as well as those with the negative control virus preparation show no evidence of mumps virus specific effects in the lateral ventricle regions. The remaining 10% of animals of the negative control virus group had very low level effects. With respect to the remaining 10% of animals of the Diluent group, it was observed that one specimen (5%) had a Grade One level effect while the other specimen may or may not have had any effects in the lateral ventricle size. As the diluent sample was free of any viral residues it is understood that the one specimen which showed a Grade One level effect was an experimental artefact.
This damage may have been caused by the needle track during I.C. inoculation or damage may have occurred to the tissue section during the experimental processing. It is also possible that the diluent medium itself might have been injected directly into the ventricular cavity causing an irreversible alteration in the size of the lateral ventricle. Many animal models note discrepancies of this nature including the macaque models of HIV. This also highlights the need for several animals to be studied in each experiment in order to minimise experimental anomalies.

4.6 **Role of positive and negative control virus preparations in the assessment of RNVTs.**

For logistical and technical reasons it was not possible to inoculate more than 60 animals per experiment in this study. One way to identify variations between individual RNVTs conducted at different time periods was to include some common samples in each test. For this reason, two preparations, one to be used as a positive control, Lo1/ Mu90, and other to be used as a negative control, SKB-JL vaccine bulk, were evaluated in each test. The combined data of the lateral ventricle size alterations (Table 3.3; Table 3.8; Table 3.9) show that of all the viruses tested the Lo1 preparation was most neurovirulent. This confirms previous results of this preparation as assessed in the monkey model by Afzal et al (1999a).

However, test to test variations were observed with respect to the scale of the enlargement of the lateral ventricle size, although overall all animals presented with almost similar effects. The majority of the samples were in Grade One to Grade Two range, only 3 RNVT's demonstrated a Grade Three effect and 2 RNVT's a Grade Four effect. The virus preparation that was used as a negative control also produced consistent data, although RNTV B, RNTV D and RNVT I showed some higher background scorings but they were observed with one or two specimen(s) only.

In summary, the use of Lo1 and SKB JL vaccine bulk has proved to be a useful choice to assess the performance of any individual RNVT against the cumulative data of the study.

4.7 **Additional Parameters**

Based on previous results published by Rubin et al (1998b) it is understood that enlargement of
the lateral ventricle following mumps virus I.C. inoculation would remain a key parameter to predict the levels of virulence that may be associated with any given strain of mumps virus. However, in this study nine other parameters were also examined with the objective that their histopathological observations may provide some further information that could be used for an accurate strain specific neurovirulence prediction in the neonatal rat model.

4.7.1 Thickness of the Cerebral Cortex.

The thickness of the cerebral cortex alters as a result of infection with MuV. During the course of this project, it was noted that this alteration seemed to be dependent on the enlargement of the lateral ventricle. It was noted that with all the viruses studied, including the positive and negative controls, as the size of the lateral ventricle increases, the size of the cerebral cortex decreases, showing an inverse relationship between the two parameters.

The viruses which showed no increase in the lateral ventricle size, such as JL MSD vaccine bulk, also failed to show any decrease in the thickness of the cerebral cortex. Viruses displaying an enlarging effect on the lateral ventricle; Lo1/Mu90, SKB Urabe Bulk Vaccine, Nt5 Urabe strain, JL5/4 vaccine sub-component, and the clinical material 99-0333, also exhibit a decreasing effect on the thickness of the cerebral cortex.

Viruses displaying a slight enlarging effect on the lateral ventricle; JL2/4 vaccine sub-component and Lo4 do not show any variation in the cerebral cortex thickness. This could be due to the fact that the enlarging effect on the lateral ventricle region is very small and did not exert significant pressure on the cerebral cortex to produce a measurable effect. However the clinical isolate, 99-0333V, had Grade One and Two level effects in the lateral ventricle but no detectable effects on the thickness of the cerebral cortex leading to the suggestion that either the correlation between the two parameters is not accurate or an insufficient number of animals were studied in the clinical material sub groups to identify any cerebral cortex variability.

Further investigations are needed in this direction.
4.7.2 The Ventricles.

Rubin et al (2000) noted that after MuV i.c. inoculation the third ventricle increases in size but the enlargement is small in comparison to the scale seen in the lateral ventricle region. The results of this study support their observations. It was noted during this study that of the viruses which had an effect on the third ventricle; SKB JL Vaccine Bulk and Lo4, the most visible effects were seen on the epithelial layer of the ventricle. This would be consistent with what is known of MuV infection; if MuV had an affect on the third ventricle, the cells surrounding the ventricle would be the first cells to show evidence of the infection. Once those cells had altered the equilibrium flow of the CSF into the ventricle, it would enlarge. As the incubation period of the virus in this study was only 30 days, it is possible that the virus needs a longer period to reach to the cells surrounding the third ventricle. It would be useful in further studies to use immuno-histo staining methods to identify the localisation and distribution of the mumps virus within various regions of the inoculated brain.

The current results are inconclusive in predicting whether or not oedema occurs before or after the ventricle starts to enlarge. It appears that in most cases; Lo1/Mu90, JL2/4, JL5/4, Nt5, FDA A, B and D strains, the oedema may have started to occur after the ventricle has begun to enlarge, this is known by the degree to which each change has occurred. The SKB Urabe Vaccine, 99-0333V and FDA A strain inoculated specimens suggest that oedema may have occurred before the ventricle starts to enlarge. These results imply that after MuV has infected the epithelial layer, the CSF accumulates in the surrounding tissue before invading the ventricle.

As infection seems to be established in the lateral ventricles of all neurovirulent viruses it is difficult to determine the course of the infection. Once the epithelial layer shows signs of infection it can be assumed that the course of infection has started, it is unclear if the grading of the epithelial layer would increases over the prolonged incubation.

Taking only the size of the lateral ventricle and the surrounding oedema into consideration, the results for the lateral ventricle suggest that the majority of viruses have the enlarging effect before oedema occurs; JL5/4 and 99-0333 are the only viruses that suggest otherwise. This
would be the more logical course of the infection.

4.7.3 Epithelial Layer

The ability of the epithelial layer to be regarded as an additional marker for mumps neurovirulence in the rat model could be considered after further investigations in which inoculated animals should be incubated for various time periods are performed. The observation of this parameter in conjunction with the observation of the enlargement of lateral ventricle size would be very useful for specimens that could not be scored unambiguously and therefore give anomalous results.

4.7.4 The Cerebellum

Viruses which showed a neurovirulent effect on the cerebellum probably display one of the two below mentioned courses of infection.
Figure 4.1: Sagittal cross-section of a normal cerebellum of a neo-natal rat brain
The first possibility is that the virus may enter the cerebellum from the adjacent cerebral cortex and infects the Molecular Layer (ML) causing cells of the ML to migrate and accumulate. The virus infection then moves to the Purkinje Cell Layer (PCL) where it causes accumulation of cells on the tips of the PCL folds in the Granular Layer (GL). If the virus is extremely neurovirulent, the cell accumulations can bud off into the GL. The GL was rarely seen to be infected in this study, but when it showed any sign of infection, it was mild.

According to the second theory the virus affects all three layers simultaneously but infection takes longer to establish in the granular matter and PCL than the molecular layer.

The former theory is more convincing as the virus likely spreads from cell to cell in the cerebral cortex, after which the virus would penetrate the ML of the cerebellum.
Figure 4.2: Sagittal cross section of the cerebellum of a neo-natal rat brain showing MuV infection.

Showing an infected cerebellum, indicating the extent that the cells in the ML migrate and aggregate, as well as the accumulation of the PCL cells. This differs from the normal...
cerebellum, Figure 4.1.

The cerebellum region could also be a good area to assess the damage caused by the virus following I.C. inoculation. Throughout this study a change in any of the layers of the cerebellum was always accompanied by a change in the size of the lateral ventricle. However, it is not speculated that cerebellum changes are a direct result of the enlargement of the lateral ventricle size, as both areas could be independently sensitive to mumps virus infection.

4.8 FDA Scoring of Samples

4.8.1 NIBSC Samples

Both scoring methods, in house as well as the one developed at FDA, produced data sets that were comparable with each other. For instance, the data produced by assigning plus/minus scores in relation to the enlargement of the size of the lateral ventricle region demonstrated that the Lo1 preparation was the most neurovirulent amongst all strains tested. This could also be concluded from the data obtained following the numerical scoring procedure. A similar correlation can be observed with the JL SKB vaccine bulk preparation that was used as a negative control throughout this study. It has been observed in this study that the JL SKB vaccine preparation is significantly more virulent than the JL MSD vaccine preparation, confirming a previous study that also observed this pattern (Afzal et al 1999b). This raises a question mark on the suitability of the JL SKB vaccine to be used as a genuine negative control. The JL SKB vaccine was selected as the negative control in this study for several reasons: A) It has not been associated with the development of meningitis in vaccine recipients, though similar could be argued for the JL MSD vaccine, B) unlike the JL MSD vaccine which is composed of two sub-variant virus populations the JL SKB vaccine is pure in its composition as it is derived from a single plaque picked virus stock. As one of the objectives of this study was to evaluate whether the neurovirulence test could be conducted in a reproducible fashion, it was vital to include a control preparation whose viral composition was relatively pure. The SKB JL vaccine bulk lot fulfilled that criterion.
Broadly, both scoring methods gave a similar conclusion; however, raw data obtained following the FDA scoring approach were user friendly for statistical analyses. It is vital that in each group a large number of animals should be inoculated to yield statistically meaningful results. The data reported in Tables 3.4, 3.7 and 3.9 indicate that the virus free diluent sample appears more virulent than the MSD JL vaccine bulk preparation which contained both JL2 and JL5 sub-variants. The higher virulence of the diluent sample is based on the observations of a total of 19 specimens, one of which had low level (+/-) effects and one had grade one level effects on the size of lateral ventricle. The remaining 17 animals did not show any histopathological abnormalities. The higher group mean percentage of lateral ventricle size shown in Table 3.9 is principally based on the reading of one sample that showed very high lateral ventricle enlargement, which was not seen with any other animals in this group. Therefore, this reading must be considered as an experimental anomaly in this study.

Overall, the consistency between various RNVTs conducted in this study was good and reproducible, both negative and positive control virus preparations played a key role in demonstrating this. The results of clinical specimen, 99-0333, and its associated tissue culture grown material, must be read with great care. This is due to the fact that only four animals were inoculated with each sample and, therefore, any experimental anomaly, as seen with the diluent inoculated group, would have had a large effect on the group average score. Further studies, using more animals, are vital to draw any final conclusion about the performance of the clinical, and low passage level, samples in the rat model in relation to other vaccine and wild type strains.

It is interesting to observe that the JL MSD vaccine bulk lot, which contained both JL2 and JL5 residues scored 0.14% on the group mean index (Table 3.9) where its plaque purified, Vero cell grown, components scored 1.0% and 4.8% respectively. This indicates that a combined version of JL2 and JL5 is less virulent than the culture grown viruses. It is widely believed that the passaging of viruses in culture induces attenuation and, therefore, should lead to a reduction in the virulence of the virus. However, this was not observed with the JL vaccine strain viruses. It
would be of further interest to note if the SKB JL vaccine which is exclusively made of the JL5 component, could also gain virulence following *in vitro* passages in Vero cells.

The Urabe vaccine, which has been definitively associated with post-vaccine aseptic meningitis in children, scored 2.7% on the group mean index (Table 3.9) which is higher than the Lo4 index (2.2%) but lower than the NT5 index (4.4%). Both Lo4 and NT5 viruses have been serially passaged in Vero cells. The Lo4 was originally isolated from a healthy child, and NT5 from a sick child, both of whom were given SKB MMR vaccine that contained the Urabe mumps virus. The effect of *in vitro* passage of the Urabe related viruses is different from that of the JL related viruses. Compared to the vaccine virus, the Lo4 virus lost virulence while the NT5 virus gained virulence, with both viruses having been passaged in Vero cells after isolation. This leads to the speculation that the neurovirulent potential of mumps vaccines is probably substrate dependent and passaging in Vero cells may enhance neurovirulence.

It is also interesting to note that the JL5 component of MSD JL vaccine, passaged in Vero cells, has a lateral ventricle size index almost similar to the index of the NT5 isolate. More interestingly, the JL5 based vaccine, SKB JL, is also 4 to 5 passages away from the MSD JL5 vaccine version, but proven to be clinically safe and has a lateral ventricle index of 0.42%. The difference between the JL5/4 and JL5 in SKB JL vaccine lies in terms of the cell substrates used for propagation. The JL5/4 was propagated in Vero while the JL5 in SKB JL vaccine was propagated in CEF cells. It is reasonable to suggest that CEF's have more attenuating effect while the primate cell line, Vero, has the opposite effect on the neurovirulence of mumps viruses in the rat model.

Based on the statistical data reported in Table 3.10 it is possible to suggest that the test could differentiate the wild type strain, Lo1, from the other strains examined in this study. The test could also distinguish the Urabe vaccine lot from the JL based vaccine lots.

4.8.2 *Classification of FDA samples*
Although the identity of viruses obtained from FDA, USA, is not known at this time, it is possible to assign them a putative designation based on their neurovirulence profiles observed in the rat model experiments at NIBSC.

As the FDA A sample displays many histopathological features that are common with the strain used as a negative control in this study it is possible to suggest that this sample may be related to or of a similar strain to the SKB JL vaccine bulk lot.

FDA B sample is more neurovirulent than the Lo1/Mu90 strain, concluding that it is either a wild type strain or a neuro-adapted strain of virus.

FDA C sample a level of neurovirulence greater than any NIBSC sample except the positive control Lo1/Mu90.

FDA D sample shows similar neurovirulence to the FDA C sample. This too may be a vaccine virus.

FDA E strain characteristics closely resemble to the negative control samples, therefore it may be a virus with no neurovirulent activity or no virus.

However, it is essential to emphasise that the above mentioned classification of FDA strains is purely a prediction, based on histopathological data of lateral ventricle size enlargement collected at NIBSC, it may differ when the actual sample codes are revealed after full data analyses by NIBSC and the FDA.
5. Conclusions

The key objective of this study was to evaluate the neonatal rat model for its ability to distinguish between various preparations of mumps viruses belonging to vaccines or wild type phenotypes. In addition, the model was also tested for its reproducibility, consistency and accuracy by using blinded samples that were exchanged between the two laboratories, NIBSC, UK, and FDA, USA. Assays were conducted in both laboratories independently using identical scoring methods. The key conclusion of this study is that all strains that were distinguishable from each other in the monkey were also distinguishable from each other in the neonatal rat model. This alone is sufficient to suggest that the future mumps neurovirulence studies could be conducted on rats rather than on monkeys. However, further work is needed to refine the methodology, in terms of the inoculation dose, study period, histopathological processing of tissues and slide readings, and in the selection of negative and positive control samples. In the current format the neonatal rat model could clearly distinguish between:

- Wild type strain and vaccine strains.
- Mumps vaccines of different strains, e.g. JL derived vs Urabe derived.
- Mixed or pure vaccine preparations of JL strain, e.g. SKB JL vs MSD JL
- The two residual components of MSD JL vaccine propagated in Vero cells from its progenitor vaccine lots.

Although several parameters of the brain were investigated in this study, no parameter other than the lateral ventricle size enlargement was considered to be a suitable predictor of mumps neurovirulence. Histopathological examination of the cerebellum region could complement the results of lateral ventricle size enlargement region and this correlation could be used to confirm ambiguous results.

Undoubtedly, the neo-natal rat model is a more convenient animal system than the macaque model for its inherent advantages related to ease, cost, management and utility of large
number of animals per experiment and, therefore, would produce statistically meaningful data. More thoughts are needed to be given to decide about the preparation intended to be used as a negative control in future investigations. The MSD JL vaccine bulk lot was least neurovirulent in this study but it lacks residual component purity. The SKB JL vaccine bulk lot is pure in its composition but it was shown to have a measure of neurovirulent effect on the lateral ventricle size of the neo-natal rat brain.

The samples used in this project are listed in the table below according to their neurovirulent activity.

Table 5.1: Summary of the neurovirulence of the specimen used in this project, in descending neurovirulent activity.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Comments</th>
<th>Percentage Mean Scoring</th>
</tr>
</thead>
<tbody>
<tr>
<td>FDA B sample</td>
<td>Unknown sample</td>
<td>24.04</td>
</tr>
<tr>
<td>Lo1/Mu90 wild-type virus</td>
<td>Isolate from parotitis case, neurovirulent in monkeys.</td>
<td>12</td>
</tr>
<tr>
<td>FDA C sample</td>
<td>Unknown sample</td>
<td>5.71</td>
</tr>
<tr>
<td>FDA D sample</td>
<td>Unknown sample</td>
<td>5.2</td>
</tr>
<tr>
<td>JL5 (x4 vero passages)</td>
<td>Component of MSD JL vaccine bulk</td>
<td>4.8</td>
</tr>
<tr>
<td>Nt5</td>
<td>Isolate from symptomatic vaccinee</td>
<td>4.4</td>
</tr>
<tr>
<td>Clinical Material 99-0333V</td>
<td>Isolated from parotitis case</td>
<td>3.5</td>
</tr>
<tr>
<td>Clinical Material 99-0333</td>
<td>As above with x1 vero passage</td>
<td>2.8</td>
</tr>
<tr>
<td>Urabe SKB vaccine bulk</td>
<td>Vaccine implicated in adverse events</td>
<td>2.7</td>
</tr>
<tr>
<td>FDA E sample</td>
<td>Unknown sample</td>
<td>2.58</td>
</tr>
<tr>
<td>Lo4 (x2 vero passages)</td>
<td>Isolate from asymptomatic vaccinee</td>
<td>2.0</td>
</tr>
<tr>
<td>FDA A sample</td>
<td>Unknown sample</td>
<td>1.3</td>
</tr>
<tr>
<td>JL2 (x4 vero passages)</td>
<td>Component of MSD JL vaccine bulk</td>
<td>1.0</td>
</tr>
<tr>
<td>SKB JL vaccine bulk</td>
<td>Vaccine with no known adverse events</td>
<td>0.42</td>
</tr>
<tr>
<td>Diluent</td>
<td>Non-neurovirulent</td>
<td>0.28</td>
</tr>
<tr>
<td>MSD JL vaccine bulk</td>
<td>Current vaccine not known to cause adverse events.</td>
<td>0.14</td>
</tr>
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6. Future Studies

In future projects, it would be useful to study the effect of passaging vaccine sub-populations and other non-neurovirulent mumps viruses in Vero and CEF cell substrates to detect any evident change in the virulence of the virus. It would also be interesting to note if the degree and nature of any change is dependent upon in which cell substrate the virus has been propagated.

This study shows that the neo-natal rat model could not distinguish between a neurovirulent and non-neurovirulent (good and bad) vaccine preparations. It also suggests that analysing events in the cerebellum may confirm or disprove ambiguous data. Immuno-histochemical staining could be used to determine the presence and localisation of the mumps virus within the brain. Molecular detection using RT-PCR approaches could also be performed for a more definitive method of virus detection.

This study also proves the need for a clearly defined positive and negative control for the neo-natal rat model before it is suggested to replace the current macaque model.

The animals used in this study were incubated for thirty days post inoculation. Using a specified time period coupled with immuno-histochemical staining, RT-PCR, or in-situ hybridisation, it may be possible to determine not only the presence of mumps virus in the neo-natal rat brain but the course of the infection it pursues.
7. Evaluation of Coded FDA Samples

The coded viral samples received from the FDA were uncovered at the *viva voce*.

Table 7.1: Summary of the uncovered virus samples received from the FDA.

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<tr>
<th>FDA Code</th>
<th>FDA Description</th>
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<tr>
<td>A</td>
<td>Jeryl Lynn unpasaged monovalent mumps vaccine. By Merck and Co</td>
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<tr>
<td>B</td>
<td>88-1961. Wild-type strain isolated from saliva of patient with parotitis and symptoms of CNS infection. Passaged twice on vero cells</td>
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<tr>
<td>C</td>
<td>Pariorix, unpasaged mumps monovalent vaccine consisting of attenuated Urabe-AM9 strain. By GlaxoSmithKline.</td>
</tr>
<tr>
<td>D</td>
<td>87-1004. Pariorix post-vaccine derived isolate, obtained from CSF of patient with vaccine-associated meningitis. Passaged twice on Vero cells</td>
</tr>
<tr>
<td>E</td>
<td>JL P3. Merck and Co’s vaccine passaged three time on vero cells</td>
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</tbody>
</table>

Table 5.1 shows the results of this study in descending neurovirulent activity. With the FDA samples showing the order B, C, D, E and A. Once this information is applied to Table 7.1, it can be seen that the FDA blind study lends weight to the rat model being used as a tool for detecting neurovirulent activity of mumps.

Table 5.1 shows that FDA B sample is the most neurovirulent of all samples, with a 24.04% scoring of the lateral ventricle size. This sample is isolated from a patient displaying symptoms of CNS infection concurrent with the study's findings. The NIBSC lateral ventricle data also shows that this sample, showed no negative samples and went as high as a ++++ grading for lateral ventricle size, the highest of all the gradings.

The FDA C sample has a mean scoring of 5.71% for lateral ventricle size, showing a degree of neurovirulent activity. It also showed 66.7% of samples grading from + to ++, with the remainder grading -/+ (Appendix I). FDA C is a discontinued urabe based vaccine called Pariorix which has been proven to exhibit CNS complications. The FDA D sample is related to the FDA C sample, it is isolated from a patient with Pariorix vaccine-associated meningitis, which could be
the reason these two samples are closely related in terms of percentage lateral ventricle size (5.71% for the former and 5.2% for the latter). The Students un-paired T-Test gives a P value of 0.972 for these two samples, suggesting a low probability of these two samples are related as a random event.

The FDA E sample exhibits a 2.58% scoring for lateral ventricle size. This is a Jeryl Lynn based vaccine and would therefore have expected the scoring to be much lower, based on observations made throughout this study. However, this vaccine has been passaged three times in vero cells, which as this study has concluded, may cause the virus to increase in neurovirulent potential. The NIBSC scoring for this sample was 85% negative with 15% grading + to ++ (Appendix I).

The FDA A sample has the smallest scoring for lateral ventricle size of all the FDA samples, which is concurrent with it being a JL vaccine from Merck and Co (1.3%). However, compared to the MSD JL vaccine that was used by NIBSC in this study, this is relatively high, 0.14% compared to 1.3%. This could be due to the composition of the JL P3 vaccine, it is unknown whether it is only one of the isolates, JL2 or JL5, or a mixture of the two as MSD JL is. The NIBSC scoring showed 94.7% of samples as negative with the remainder grading + to ++.

The samples were organised to range from most neurovirulent activity to the least (Table 5.1), the uncovering of these samples concurs with that list. Thus these blinded samples have lent weight to strengthen the belief that the rat model acts as a reliable indicator of the neurovirulent activity of a mumps virus based vaccine or sample.
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168


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Appendix I
Tabulated data of each RNVT including positive and negative control groups.
RNVT B: Positive and Negative Controls and SKB Urabe Bulk Vaccine

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**Cerebellum**

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Appendix II.

FDA Scoring of NIBSC samples

Table 1: Lateral Ventricle size of animals inoculated with the Lo1/Mu90 Positive Control Virus

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<tr>
<th>Animal Designation</th>
<th>Area of Brain covered by Lateral Ventricle %</th>
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Mean Value: 12.04
Table 2: Lateral Ventricle size of animal inoculated with the SKB JL Bulk Vaccine

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<td>13</td>
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<tr>
<td>391</td>
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</table>

Mean Value: 0.24%
Table 3: Lateral Ventricle size of animals inoculated with SKB Urabe Bulk Vaccine.

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</tr>
<tr>
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Mean Value: 2.7%
Table 4: Lateral Ventricle Size of animals inoculated with Lo4 Urabe isolate.

<table>
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<td>149</td>
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<td>153</td>
<td>1.45</td>
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<td>154</td>
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<tr>
<td>155</td>
<td>-</td>
</tr>
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Mean Value: 1.96% (2%)
Table 5: Lateral ventricle Size of Animals inoculated with Nt5 Urabe virus.

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<td>39</td>
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Mean Value : 4.37%
Table 6: Lateral Ventricle Size of animals inoculated with the JL MSD Bulk Vaccine.

<table>
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Mean Value : 0.14%
Table 7: Lateral Ventricle Size of animals inoculated with JL2 sub-component

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Mean Value : 1%
Table 8: Lateral Ventricle Size of animals inoculated with the JL5 sub-component

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<th>Area of Brain Covered by the Lateral Ventricle %</th>
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Mean Value: 4.8%
Table 9: Lateral Ventricle size of animals inoculated with the diluent.

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<tr>
<td>411</td>
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Mean Value: 0.28%
Table 10: Lateral Ventricle size of animals inoculated with the Clinical Material 99-0333

<table>
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<td>284</td>
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</table>

Mean Value : 2.8%

Table 11: Lateral Ventricle size of animals inoculated with the Clinical Material 99-0333V

<table>
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<th>Area of Brain Covered by the Lateral Ventricle %</th>
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<tr>
<td>288</td>
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</table>

Mean Value : 3.48%
Table 12: Lateral Ventricle size of animals inoculated with the FDA A Sample.

<table>
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Mean Value: 1.3%
Table 13: Lateral Ventricle size of animal inoculated with the FDA B Sample

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</tr>
</thead>
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Mean Value: 24.04%
Table 14: Lateral Ventricle size of animals inoculated with the FDA C Sample

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Mean Value : 5.71%
Table 15: Lateral Ventricle size of the animals inoculated with the FDA D Sample

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</table>

Mean Value : 5.2%
Table 16: Lateral Ventricle size of the animals inoculated with FDA E Sample

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Mean Value: 2.58%