

**The Immunogenic Epitopes of the Glycoproteins  
of Human Metapneumovirus**

**Alison Beth Tedcastle**

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## **Dedication**

I dedicated this thesis to my family, who throughout have been a constant source of support and encouragement.

## **Declaration**

This thesis contains no material which has been accepted for any other degree in any university. Unless otherwise stated, all of the work presented was performed by me at the University of Newcastle upon Tyne under the supervision of Professor Geoffrey Toms.

Alison Beth Tedcastle

## Abstract

Recently discovered in 2001, human metapneumovirus (HMPV) is a member of the *Paramyxoviridae* family and is a major cause of respiratory tract infections in infants and young children as well as the elderly and immunocompromised.

In the related pneumovirus RSV, the two major surface glycoproteins, F and G are protective antigens in animal models although F is highly conserved and G highly variable. In this study, the equivalent glycoproteins of HMPV, F and G, were cloned into vaccinia virus to allow expression of the individual proteins. These recombinants were utilised for the generation of both monoclonal and glycoprotein specific polyclonal antibodies. Immunofluorescence studies revealed that the anti-F protein specific antibodies were cross reactive between both sub-groups and that anti-G antibodies were to a lesser extent, also cross reactive. These antibodies were also shown to neutralise homologous virus. Whilst anti-F protein antibodies also neutralised a heterologous strain of HMPV, so did anti-G antibodies directed towards a sub-group B but not a sub-group A strain. Western blotting with F and G glycoprotein specific anti-sera was unsuccessful due to high levels of non-specific reactivity in the sera.

The generation of monoclonal antibodies towards the G glycoprotein was attempted by means of a novel screening system using inactivated recombinant vaccinia virus. However, due to the non-specific reactivity of the hybridomas with vaccinia virus, only one anti-G antibody was isolated along with two anti-F MAbs and one antibody directed towards an internal HMPV protein. Further characterisation of this antibody, by western blotting, indicated it was directed towards the phosphoprotein.

The third surface glycoprotein of HMPV, SH, is larger than the equivalent in RSV and unlike the latter, may also play a role in protective immunity. Attempts to clone the HMPV SH gene identified several mutations in the sequence resulting in truncation of all or the majority of the luminal domain of the proteins arising on adaption to replication in cell culture. SH glycoprotein specific antibodies generated against the recombinant vaccinia virus expressing the mutated SH protein of the B1

strain of virus were cross reactive with an A2 strain in immunofluorescence studies and also neutralised wild type HMPV.

The tendency of the virus to mutate on adaptation to replication in cell culture frustrated attempts to establish an animal model. In mice, whilst low passage virus, with a mixed population of wild type and mutant virus, resulted in a productive infection, high passage virus, with no functional SH glycoprotein produced an abortive infection with evidence of genome replication and transcription but no release of infectious virus. However, in challenge studies with mutant viruses mice immunised with HMPV were protected when challenged with the homologous strain, however, immunisation of mice with recombinant vaccinia virus expressing the G glycoprotein showed no signs of protection against challenge suggesting that in this animal model, the G glycoprotein is not a protective antigen.

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## List of abbreviations

16HBE140	human bronchial epithelial cells
aa	amino acid
AGM	African green monkey
AMPV	avian metapneumovirus
APS	ammonium persulfate
BEI	Binary ethylenimine
BAL	bronchoalveolar lavage
bp	base pair
BuDR	5-Bromo-2-deoxyuridine
BSA	bovine serum albumin
°C	degrees Celsius
cDNA	complementary deoxyribonucleic acid
CO <sub>2</sub>	carbon dioxide
CPE	cytopathic effect
Cp	cold-passage
Ct	cycle threshold
CTL	cytotoxic T lymphocytes
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
<i>E. coli</i>	Escherichia coli
EDTA	ethamine-diamine tetra acetic acid
ELISA	enzyme linked immunosorbent assay
EMEM	Eagle minimum essential medium
EMC	encephalomyocarditis virus independent ribosomal entry site
ER	endoplasmic reticulum
F	fusion glycoprotein
FCS	foetal calf serum
f.f.u.	focus forming units
FFA	fluorescent focus assay
FITC	fluoresceine isothiocyanate

G	attachment glycoprotein
g	gram
GAG	glycosaminoglycan
GE	gene end
GM	growth medium
GS	gene start
H <sub>2</sub> O <sub>2</sub>	hydrogen peroxide
HBSS	Hanks' balanced salt solution
HCl	hydrochloric acid
HMPV	human metapneumovirus
HPIV	human parainfluenza virus
HRP	horse radish peroxidase
HRSV	human respiratory syncytial virus
H <sub>2</sub> SO <sub>4</sub>	sulphuric acid
IF	immunofluorescence
IFN	interferon
Ig	immunoglobulin
IVIG	intravenous immunoglobulin
Kbp	kilo base pair
KDa	kilo Dalton
L	large polymerase protein
M	matrix protein
MAB	monoclonal antibody
MCS	multiple cloning site
ml	millilitre
MM	maintenance medium
M.O.I.	multiplicity of infection
mRNA	messenger ribonucleic acid
N	nucleoprotein
NDV	Newcastle disease virus
NPS	nasopharyngeal secretion
NS	non-structural
OD	optical density
OPD	o-phenylenediamine

ORF	open reading frame
P	phosphoprotein
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBST	PBS tween
PCR	polymerase chain reaction
PFU	plaque forming units
PTF	PBS tween with 10% foetal calf serum
PVDF	polyvinylidene fluoride
qT-PCR	quantitative polymerase chain reaction
RAP-PCR	random amplification polymerase chain reaction
RNA	ribonucleic acid
RNAP	ribonucleic acid polymerase
RNP	ribonucleoprotein
rpm	revolutions per minute
RT-PCR	reverse transcription polymerase chain reaction
RTI	respiratory tract infection
RSV	respiratory syncytial virus
RNP	ribonucleoprotein
TEMED	N, N, N, N – tetramethylethylene-diamine
ts	temperature sensitive
tMK	tertiary monkey kidney cells
SAF	serum and antibiotic free
SAM	sheep anti - mouse
SDS	sodium dodecyl sulphate
SH	small hydrophobic glycoprotein
UV	ultraviolet light

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## Chapter 1: Introduction

### 1.1 Discovery of human metapneumovirus

In 2001 in the Netherlands, van den Hoogen *et al* reported the isolation of a new respiratory virus from nasopharyngeal secretions of 28 children, over a 20 year period, suffering from respiratory tract infections (RTIs). All but one of these patients were under the age of 5 years and 13 children were under 12 months old. These children presented with a range of symptoms similar to those caused by respiratory syncytial virus (HRSV) including bronchiolitis and pneumonia.

Seroprevalence studies on a range of human sera, using indirect immunofluorescence (IF), indicated that 25% of children aged between 6 and 12 months had antibodies to the virus and revealed that by the age of 5 years, nearly all children had been exposed to human metapneumovirus (HMPV). Seventy-two serum samples taken from individuals in 1958 were all positive by IF, suggesting that the virus had been circulating in the human population for at least 40 years.

Using negative contrast electron microscopy, the group demonstrated the presence of paramyxovirus-like particles between 150 and 600nm in diameter with external spikes 13 to 17nm in length. The unidentified virus replicated in a slow, trypsin dependent manner in tertiary monkey kidney cells (tMK), did not haemagglutinate erythrocytes and could be inactivated with chloroform.

Ferrets and guinea pigs inoculated intranasally with viral isolates seroconverted, allowing their sera to be used for further IF studies. Whilst the tMK cell cultures infected with viral isolates tested positive with this sera, cells infected with human parainfluenza viruses (HPIV) 1 to 4, HRSV and influenza viruses A and B did not.

Reverse-transcription polymerase chain reaction (RT-PCR) performed on 15 of the isolates using primers specific for paramyxoviruses HPIV 1-4, mumps virus, measles virus, HRSV, simian virus type 5, Sendai virus and Newcastle disease virus (NDV) did not react with the isolates, thus suggesting the isolates were not closely related to these viruses.

Using random amplification PCR (RAP-PCR), 20 fragments specific to the isolates were sequenced. Ten of these fragments were matched in a BLAST search to avian metapneumovirus (AMPV) N, M, F and L gene sequences. Further sequencing using primers designed against the fragments revealed an absence of the 3' non structural genes and positioned the M gene adjacent to the F gene in the open reading frame. Analysis of the amino-acid sequences of N, M, F and L genes showed far greater homology with AMPV in comparison to HRSV. Phylogenetic analysis suggested the new virus was most closely related to AMPV C than the other three serotypes of AMPV and the possibility of two sub-groups of HMPV due to the genetic variation.

Since there was an apparent relationship between HMPV and AMPV, van den Hoogen *et al* investigated infection in birds (turkeys and chickens) and monkeys (cynomolgus macaques) and found signs of clinical disease and virus replication in the monkeys but not the birds suggesting this virus is a primate pathogen that produces a respiratory disease (van den Hoogen *et al.*, 2001).

On this basis, the group tentatively suggested the name human metapneumovirus, which was subsequently confirmed by the International Committee for the Taxonomy of Viruses (Fauquet *et al.*, 2005).

## **1.2 Classification of human metapneumovirus**

After analysis of the sequence data, HMPV has been classified in the order of *Mononegavirales* within the family of *Paramyxoviridae*. This is divided into two sub-families; *Paramyxovirinae* and *Pneumovirinae* of which HMPV belongs within the *Metapneumovirus* genus (Figure 1.1) (Fauquet *et al.*, 2005).

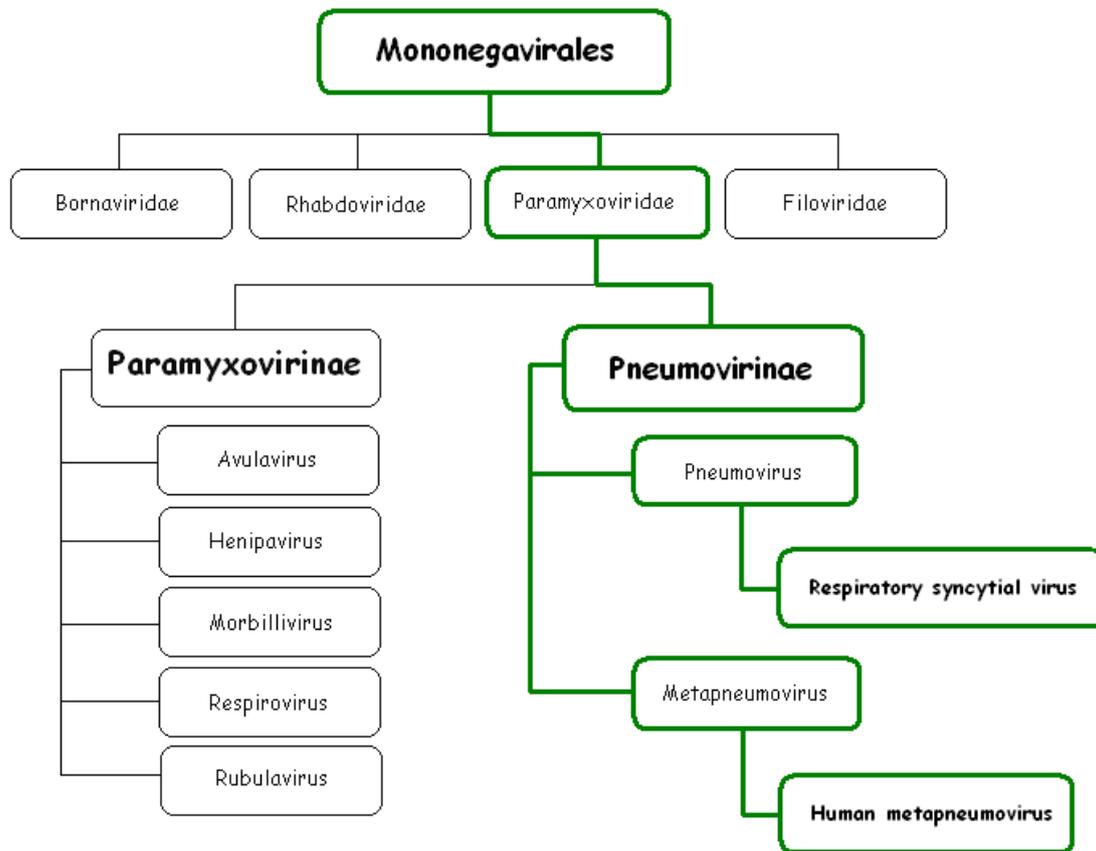


Figure 1.1 Taxonomy of Mononegavirales family (Fauquet *et al.*, 2005)

### 1.3 The genomes of the *Paramyxoviridae*

The genomes of the *Paramyxoviridae* can contain six to ten genes, five of which are structurally and functionally similar in all viruses. These are the nucleocapsid protein (N), a phosphoprotein (P), the RNA dependent RNA polymerase (L), the matrix protein (M) and the F glycoprotein (F). The N proteins association with the L and P proteins, which together form the ribonucleoprotein (RNP), is responsible for initiating transcription of the RNA genome to produce mRNAs. Situated between the RNP and the viral envelope is the M protein which is thought to be important in virion architecture and is released from the core during viral entry, (Lamb and Parks, 2007; van den Hoogen *et al.*, 2002; van den Hoogen *et al.*, 2001). All viruses possess two glycosylated protein spikes on the surface of the membrane involved in mediating viral attachment and fusion of viral and host cell membranes (Lamb and Parks, 2007).

## 1.4 The genomes of the human pneumoviruses

Due to the recent isolation of HMPV in 2001, almost all that is known about the proteome and mechanisms of replication is by analogy with human respiratory syncytial virus (HRSV). Therefore I shall review the mechanism of infection in HRSV alluding where appropriate to information relating to HMPV.

Both HRSV and HMPV have a single stranded, non-segmented, negative sense RNA genome which is tightly encapsidated by the nucleocapsid protein. Genomic RNA serves two purposes, the first as a template for the production of mRNA transcripts and the second, as a template for the synthesis of the antigenome; necessary for replication (Collins and Crowe, 2007).

At the 3' end of the HRSV genome, a 44-nucleotide leader region (41 nucleotides in HMPV and AMPV) is present containing promoters responsible for directing synthesis of the mRNAs and antigenome. A 155-nucleotide trailer region (179 nucleotides in HMPV) located at the 5' end contains the antigenome promoter, both of which are essential for replication (Melero, 2007; Mink *et al.*, 1991; van den Hoogen *et al.*, 2002).

The negative sense RNA encodes ten genes, which in turn translate into eleven structural and non-structural proteins (Collins and Crowe, 2007) (see Figure 1.2). Each gene is separated by a small variable intergenic region, with the exception of the M2 and L genes, which overlap by 68 nucleotides. Apart from the M2 gene, which contains two overlapping open reading frames M2-1 and M2-2, each gene contains a single major open reading framing that is transcribed to produce a distinctive protein (Melero, 2007).

The genome of HMPV lacks the non-structural proteins NS-1 and NS-2 of HRSV (Collins and Crowe, 2007) and as a result, encodes eight genes which translate into nine proteins. Consequently the genome of HMPV is slightly smaller measuring 13,335bp (CAN97-83, Genbank accession number AY297749) compared to 15,222bp (HRSV A2, Genbank accession number M74568) in HRSV. Furthermore, the gene

order differs slightly with the F-M2 gene pair of HRSV located closer to the 5' end of the genome (Figure 1.2).

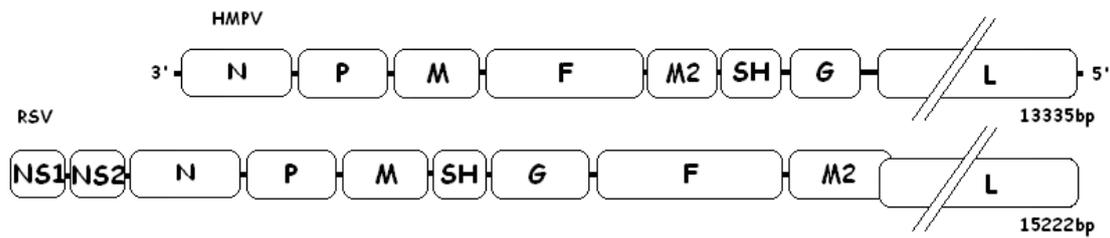


Figure 1.2 Gene order of HMPV and HRSV

### 1.5 The virion structure of the human pneumoviruses

The HRSV virions have a pleomorphic nature visualised as irregular spherical particles ranging in diameter from 150 to 300nm and long filamentous forms, 60 to 100nm and up to 10µm in length (Bachi and Howe, 1973; Collins *et al.*, 2001). The virion of HMPV is similar ranging in diameter from 150 – 350 nm and is also present in both pleomorphic and filamentous forms (Peret *et al.*, 2002).

Morphologically, the virion of both HRSV and HMPV consist of a helical nucleocapsid surrounded by a lipid envelope, which is derived from the plasma membrane of the host cell (Lamb and Kolakofsky, 2001). Embedded within this membrane are three transmembrane surface glycoproteins F, G and SH. These proteins are visualised as short ‘spikes’ that extend roughly 15nm from the particles and mediate viral attachment and entry into host cells (Collins and Crowe, 2007; Peret *et al.*, 2002).

Concealed within the lipid envelope is the viral genomic RNA tightly encapsidated by the N protein. This nucleocapsid is additionally associated with components of the viral RNA-dependent RNA polymerase (L, P and M2-1 proteins). The matrix protein is situated between the nucleocapsid and the outer envelope and is believed to form a layer on the internal surface of the membrane (Collins and Crowe, 2007).

## **1.6 Virus replication**

### **1.6.1 Virus attachment and cell entry**

Infection is initiated upon binding of the virus' attachment protein to the cell surface receptor, which in case of HRSV is thought to be glycosaminoglycans (GAGs), namely heparin sulphate and chondroitin sulphate B (Hallak *et al.*, 2000a). However, it is not clear whether this is a characteristic of wild virus or is limited to cell culture adapted strains (Hallak *et al.*, 2007). GAGs are unbranched polysaccharides consisting of repeating disaccharide sub-units located on the surface of the cell membrane in the extracellular matrix (Melero, 2007). Identification of two clusters of positively charged amino acids on the G glycoprotein of HMPV are thought to represent heparin binding domains. In addition, studies demonstrated soluble GAGs were able to inhibit HMPV infection indicating an important role for the G protein and cellular GAGs during HMPV infection (Thammawat *et al.*, 2008).

Upon attachment of the virus to the cell surface receptor, fusion of the viral and cell plasma membrane ensues at neutral pH, mediated by the F glycoprotein (Smith *et al.*, 2009). However, recent studies have indicated that some strains of HMPV may utilise the endosome pathway for entry into the host cell. The low-pH environment encountered in the endosomes may trigger the F proteins conformational change that results in membrane fusion and entry (Smith *et al.*, 2009). Schowalter *et al* (2009) demonstrated that pre-treatment of cells with chlorpromazine, an inhibitor of clathrin-mediated endocytosis, conferred protection against the virus. In addition, blocking the final step in the vesicle formation of clathrin mediated endocytosis was highly effective at blocking HMPV infection by up to 90%. Furthermore, studies by Herfst *et al* (2008b) and Schowalter *et al* (2006) have shown certain strains of HMPV to favour fusion at low pH suggesting a role for endosomal entry.

### **1.6.2 Transcription**

As a result of membrane fusion, the viral nucleocapsid is released into the cytoplasm of the host cell, where the entire process of RNA synthesis occurs. Early in virus infection, the viral RNA polymerase is restricted to the production of mRNA transcripts allowing the accumulation of viral proteins (Lamb and Kolakofsky, 2001).

Studies with minireplicon systems confirmed that the polymerase does not enter the genome at internal genes but instead transcribes in a sequential start stop manner, responding to gene start and gene end signals that flank each gene. This is common to other *Mononegavirales* (Dickens *et al.*, 1984; Kuo *et al.*, 1997; Lamb and Kolakofsky, 2001).

The gene end (GE) signal, located at the 3' end of the upstream gene is a semi-conserved 12-13 nucleotide sequence, responsible for the polyadenylation and termination of the mRNA. This motif is the same as that of HMPV (AGTTAnnnAAAA) (Biacchesi *et al.*, 2003; Kuo *et al.*, 1997). The gene start (GS) signal positioned at the 5' end of the downstream gene, is a highly conserved 9 to 10 nucleotides (GGGGCAAAT[A/T]) that directs the addition of a guanylated and methylated cap to the nascent mRNA and initiates transcription (Melero, 2007). The first 10 of the 16 nucleotides that make up the GS signal of HMPV (GGGACAAnTnnnAATG) share some resemblance to the gene start motif of HRSV. However, instead of the first nucleotide representing the mRNA start site, as seen in the GS sequence of HRSV (Kuo *et al.*, 1997), an unusual feature of the related GS motif of HMPV is the presence of an ATG start codon at positions 14-16, which initiates transcription (Biacchesi *et al.*, 2003). Separating each gene lies a non-transcribed transgenic region varying in length from 1 to 58 nucleotides (up to 190 nucleotides for HMPV). The gene-end of an upstream gene, transgenic region and gene-start region of a downstream gene are collectively referred to as the gene junction.

The viral RNA polymerase (RNAP) enters the genome at the single promoter located at the 3' end and commences transcription at the gene start sequence of the primary gene. This is the NS1 gene for HRSV and the N gene for HMPV. As it travels downstream, newly synthesised individual mRNAs are released by termination of the polymerase at the gene end signals. The addition of a methylated 5' guanine cap to each mRNA transcript assures its translation by the cell ribosomes (Melero, 2007). Upon termination of transcription at the gene end signal, the RNAP appears to remain attached to the template as it migrates across the transgenic region before reinitiating transcription of the downstream adjacent gene. However, the polymerase is inclined to dissociate from the template before reinitiation, leading to a gradient of expression,

with the genes closest to the 3' end transcribed more abundantly than those at the 5' end (Collins and Wertz, 1983). Gene expression is also controlled by the variable efficiency of transcription termination at the gene junctions resulting in read-through transcription creating polycistronic mRNAs. These read through messages contain two or more genes together with their intergenic sequences (Dickens *et al.*, 1984) and can account for up to 10% of total mRNA. Since only the first gene of a polycistronic transcript is translated, it reduces the expression of the protein encoded by the next downstream gene (Collins and Crowe, 2007; Melero, 2007).

### **1.6.3 Replication**

The accumulation of core proteins, namely the M2-2 protein, modulates the switch from transcription to replication (Bermingham and Collins, 1999). The availability of the N protein, however, does not alter the balance between mRNA or antigenome synthesis as is speculated for other members of the *Mononegavirales* (Fearn's *et al.*, 1997). In HRSV, despite the extensive overlapping of the transcription and replication promoters at the 3' end, they are not identical in sequence, suggesting that the polymerase complex may differ dependent on the commitment to transcription or replication (Fearn's *et al.*, 2002). Initiation of replication instigates the polymerase to disregard the gene junction signals as it travels from the 3' to 5' end and commit to the production of a full length antigenome which is complementary to the negative sense genome. The antigenome tightly assembles with the N protein (Kuo *et al.*, 1997) and its sole function is to act as a template to direct synthesis of genomic RNA, which is initiated from the antigenome promoter. The generation of negative strand genomes can subsequently be utilised as templates for mRNA synthesis, for the production of antigenomes or to be packaged into progeny virions (Lamb and Parks, 2007).

### **1.6.4 Virion assembly and release**

Viral components assemble on the apical surface and pinch off by the budding process to release virus particles. Like other *Paramyxoviridae*, HRSV is thought to exploit cytoskeletal elements in its replicative cycle. Evidence suggests that both actin and microtubules play an important role in HRSV assembly and release (Kallewaard *et al.*, 2005). Viral components are transported to the plasma membrane by the exocytic pathway, mediated by the matrix protein. Upon assembly, the virions bud out

acquiring the host cell derived lipid membrane and pinch off releasing the newly formed progeny (Lamb and Parks, 2007).

## 1.7 Genetic variation of HMPV

Similar to that of HRSV, phylogenetic analysis has revealed HMPV to be divided into two major genetic sub-groups, A and B, which can further be divided into lineages 1 and 2 (Bastien *et al.*, 2003; Biacchesi *et al.*, 2003; Galiano *et al.*, 2006; Peret *et al.*, 2002; Stockton *et al.*, 2002; van den Hoogen *et al.*, 2001), based on the extensive variability of the G glycoprotein (Endo *et al.*, 2008; Ishiguro *et al.*, 2004; Padhi and Verghese, 2008; Peret *et al.*, 2004; van den Hoogen *et al.*, 2004). Each genetic lineage is thought to co-circulate within the population with no geographic clustering where epidemics occur mainly in the winter and early spring (Boivin *et al.*, 2004; Ludewick *et al.*, 2005; van den Hoogen *et al.*, 2004; Vicente *et al.*, 2006). Within each epidemic the prevalent strain differs and the lack of co-circulation of all four lineages in the same year suggests a mechanism for evading pre-existing immunity (Agapov *et al.*, 2006; Boivin *et al.*, 2004; Ingram *et al.*, 2006; Ludewick *et al.*, 2005).

Sequence homology between the two sub-groups of HMPV (A and B), across the entire proteome, is 80% with a higher intra-sub-group sequence homology of 92%. This is attributable to the most conserved proteins, including the nucleocapsid protein and the F glycoprotein which share 85-94% amino acid homology between sub-groups. Extensive variation in the SH and G glycoproteins results in 59% and 37% homology respectively (Biacchesi *et al.*, 2003).

Extensive genetic variation at both nucleotide and amino acid level in the G glycoprotein as a consequence of nucleotide substitutions, insertions and the use of alternative stop codons results in a high degree of variability in the extracellular domain. This is postulated to be as a consequence of immunological pressure and is a strong indicator of positive selection (Bastien *et al.*, 2004; Ishiguro *et al.*, 2004; Ludewick *et al.*, 2005; Padhi and Verghese, 2008; Peret *et al.*, 2004; van den Hoogen *et al.*, 2004). In contrast, the lack of variation in the conserved F glycoprotein may reflect both structural and functional constraints and account for the extensive cross-

lineage neutralisation of antibodies to HMPV (Skiadopoulos *et al.*, 2004; van den Hoogen *et al.*, 2004).

## **1.8 Viral proteins**

### **1.8.1 The non-structural proteins of HRSV**

The presence of two non-structural proteins, NS1 and NS2, are unique to HRSV and are located at the 3' end of the genome. Location and the concept of transcription gradient would suggest they are the most abundantly transcribed proteins in the genome yet they are only detected in trace amounts (Collins and Crowe, 2007).

One of their roles is to inhibit the induction of interferon  $\alpha / \beta$  by inhibiting phosphorylation and nuclear translocation of the interferon regulatory factor 3, produced in response to viral infection (Collins and Crowe, 2007).

### **1.8.2 The nucleocapsid proteins**

#### **1.8.2.1 The nucleocapsid protein (N)**

The N protein of HRSV tightly encapsidates the viral RNA genome and antigenome to form a helical structure and together with the P and L protein form the highly stable, RNase resistant RNP complex. The association of the N protein with the viral RNA is vital for recognition by the viral RNA dependent RNA polymerase, essential for both transcription and replication (Melero, 2007).

It was originally proposed that the cytoplasmic concentration of the N protein available for encapsidation might direct the switch from transcription to replication as suggested in other *Mononegavirales* (Lamb and Parks, 2007). Contradictory evidence indicates that whilst the increased levels of N protein amplified RNA replication in the mini-genome system, the balance between transcription and RNA replication remained invariable (Fearn *et al.*, 1997). This suggests that the N protein may influence levels of replication but does not modulate the switch between mRNA and antigenome synthesis, a proposed function of the M2-2 protein (Bermingham and Collins, 1999).

The N terminal end of the N protein is relatively conserved among members of the *Mononegaviradae*. Barr *et al* (1991) identified three regions of high homology in sequence and secondary structure. Across these regions HMPV shares 99.3% sequence homology with AMPV-C and 78% with HRSV (Li *et al.*, 1996; van den Hoogen *et al.*, 2002). These regions are thought to be involved in the interaction with genomic RNA and to modulate self assembly. The variable C terminal end of the protein is required for interaction with the phosphoprotein (Karlin *et al.*, 2003) and the matrix protein during viral assembly (Barr *et al.*, 1991).

The N protein of HMPV is 394 amino acids in length and has a molecular mass of 43.5KDa similar to that of other pneumoviruses but smaller than that of other members of the *Paramyxoviridae* family. Within each sub-group of HMPV, the N protein shares 93-100% nucleotide sequence homology, whilst there is 85-86% nucleotide identity between sub-groups (Barr *et al.*, 1991; Bastien *et al.*, 2003; van den Hoogen *et al.*, 2002). The HMPV N protein shares only 42-44% amino acid identity with that of HRSV N and 69-89% with the closely related AMPV-C where both polyclonal and monoclonal antibodies to the conserved region of AMPV N cross react with HMPV N protein (Alvarez *et al.*, 2004b).

### **1.8.2.2 The phosphoprotein (P)**

The 241 amino acid phosphoprotein of HRSV is smaller than its *Paramyxovirinae* counterparts with which it shares no sequence similarity (Collins *et al.*, 2001). It is extensively phosphorylated, primarily at serine residue 232. Minor phosphorylation sites involve serine residues 116, 117, 119 and 237 (Barik *et al.*, 1995).

The role of phosphorylation remains elusive. Non-phosphorylated P protein expressed from bacteria could not support full-length transcription suggesting that phosphorylation has a role in stabilising the polymerase complex (Dupuy *et al.*, 1999). However, the removal of phosphorylation at the identified residues above had a minimal effect on virus replication and has been postulated to play a role in virus budding as apposed to viral replication (Lu *et al.*, 2002).

The paramyxovirus P protein is an essential part of the RNP complex and the C terminus provides the bridge that links the L protein with the nucleoprotein

encapsidated RNA during transcription. During replication, the N terminal domain of P is believed to prevent N protein aggregation and therefore ensures efficient virion assembly (Lamb and Parks, 2007).

The phosphoprotein of HMPV is composed of 294 amino acids with a molecular mass of 32.5KDa comparable with other pneumoviruses (Bastien *et al.*, 2003; Biacchesi *et al.*, 2003; van den Hoogen *et al.*, 2002). With regard to the P protein, HMPV members of the same sub-group share 91-100% nucleotide sequence identity, compared to 78-79% identity between the sub-groups, with the majority of amino acid substitutions observed in the amino-proximal terminal of the protein (Bastien *et al.*, 2003; Ishiguro *et al.*, 2004).

Similar to that of AMPV-C and HRSV, the P protein of HMPV lacks any cysteine residues. A high number of glutamate residues in the C terminal is a common feature shared with all pneumoviruses with 100% similarity to AMPV-C and 81% to HRSV. It has been postulated that this common region may be involved in RNA synthesis or maintaining the structural integrity of the nucleocapsid complex (Bastien *et al.*, 2003; Ling *et al.*, 1995; van den Hoogen *et al.*, 2002).

### **1.8.2.3 M2 gene**

Uniquely present in all members of the *Pneumovirus* subfamily, the HMPV M2 gene encodes two overlapping ORFs, which are expressed as two separate proteins. ORF 1 encodes the M2-1 protein measuring 187 amino acids in length (184 amino acids for HRSV) and the second ORF, M2-2, encodes a protein of 71 amino acids (83-90 amino acids for HRSV) both of which are involved in RNA synthesis (Bermingham and Collins, 1999; Buchholz *et al.*, 2005; Collins and Crowe, 2007; van den Hoogen *et al.*, 2002).

#### **1.8.2.3.1 M2-1**

The M2-1 protein of HRSV is an internal protein that co-localises with the N and P proteins to form cytoplasmic inclusion bodies (Collins and Crowe, 2007). It appears to play an important role in the process of viral transcription of mRNAs. It displays processivity and anti-termination activities, essential functions in the production of

full length mRNA transcripts. In the absence of M2-1, the viral polymerase terminates prematurely, thus processivity of M2-1 prevents the polymerase from stalling during transcription, resulting in synthesis of full length mRNAs and increasing the concentration of polymerase available to genes downstream (Collins *et al.*, 1996). The anti-termination function inhibits the polymerase from terminating at the gene end signals allowing read-through of the intergenic regions and resulting in the production of polycistronic mRNAs (Collins *et al.*, 1996; Fearn and Collins, 1999). Despite the synthesis of read-through transcripts, the M2-1 protein appears to play no essential role in RNA replication as the production of antigenomes is unaffected by the presence or absence of the M2-1 protein (Fearn and Collins, 1999).

The N terminus of M2-1 is the most conserved region and there are three cysteine residues within the first 30 amino acids. This is a common feature found in zinc binding proteins and the Cys/His motif found in the M2-1 of all pneumoviruses appears to have functional importance (Buchholz *et al.*, 2005; Collins and Crowe, 2007; van den Hoogen *et al.*, 2002).

The HMPV M2-1 protein reveals 84% sequence identity with that of AMPV-C and 35-36% with HRSV (van den Hoogen *et al.*, 2002). Studies involving the production of recombinant HMPV where the M2-1 ORF had been silenced indicated that the M2-1 protein is not essential for virus replication *in vitro*, unlike the M2-1 protein of HRSV, but is required for replication and the production of virus neutralising antibodies in hamsters (Buchholz *et al.*, 2005) suggesting M2-1 is essential for replication *in vivo*.

#### **1.8.2.3.2 M2-2**

The second ORF, M2-2 overlaps M2-1 in a conserved location and is thought to play an important role in genome synthesis by mediating the switchover from transcription to replication. Ablation of HRSV M2-2 expression resulted in reduction of both genomic and anti-genomic RNA and an accumulation in transcription products (Bermingham and Collins, 1999). The accumulation of mRNAs during transcription results in inhibition of mRNA synthesis and renders the nucleocapsid inactive before the formation of mature virions (Collins *et al.*, 1996).

M2-2 is an accessory protein since mutants lacking M2-2 are viable in cell culture (Bermingham and Collins, 1999). However, silenced M2-2 virus is highly attenuated *in vivo* yet immunogenic and protective suggesting M2-2 negative mutants may be good candidates for a potential live attenuated vaccine (Teng *et al.*, 2000).

Similar, studies involving the ablation of the M2-2 ORF of HMPV discovered uncompromised growth in cell culture involving an increase in transcription although a reduction in RNA replication was not evident (Buchholz *et al.*, 2005; Schickli *et al.*, 2008). However, contradictory evidence suggests that the M2-2 protein is inhibitory of both transcription and replication in the mini-genome system (Kitagawa *et al.*, 2009). Unexpectedly, expression of the M2-2 ORF of HMPV was completely unaffected when M2-1 was silenced, in contrast with HRSV, where the expression of M2-2 is dependable on the stop-restart translation of the cellular ribosomes exiting the M2-1 ORF (Buchholz *et al.*, 2005). Concluding, the function of both HMPV M2-1 and M2-2 may be quite different to that of the HRSV counterparts.

#### **1.8.2.4 Large polymerase protein (L)**

The L protein of the pneumoviruses is thought to be the major component of the viral RNA-dependent RNA polymerase complex involved in the synthesis of all viral RNA. Situated at the 5' end of the genome, the L protein is usually found in low abundance due to the effect of the transcription gradient (Collins and Crowe, 2007). Enzymatic activities such as methylation, capping and polyadenylation are all attributed to the L protein. Six conserved domains identified in other negative sense RNA viruses (Poch *et al.*, 1989) have been identified in the HMPV L protein and are thought to include four core polymerase motifs which are vital in polymerase function.

The HMPV L gene encodes a 2005 amino acid protein and has 64% sequence identity with the L gene of AMPV-A and 44% with HRSV. HMPV L proteins within virus strains of the same HMPV sub-group have a nucleotide sequence homology of 95% compared with 84% homology between sub-groups (Biacchesi *et al.*, 2003; van den Hoogen *et al.*, 2002).

#### **1.8.2.5 Matrix protein (M)**

The M protein of HRSV is non-glycosylated and comprises of 256 amino acids which is smaller than matrix proteins of other paramyxoviruses with no sequence homology (Collins and Crowe, 2007).

The non-glycosylated M protein of HRSV localises at the plasma membrane and is considered to have a central role in the organisation of viral morphogenesis via interaction with the cytoplasmic tails of the membrane proteins, the lipid bilayer and the nucleocapsid (Collins and Crowe, 2007). The basic nature of the protein allows interactions with acidic residues in the N protein facilitating assembly and inhibiting transcription late in the virus life cycle prior to encapsidation (Ghildyal *et al.*, 2002). In addition, the presence of the M protein in the host cell nucleus is thought to inhibit host cell transcription by the down regulation of some host cell genes, facilitating viral transcription early in infection (Ghildyal *et al.*, 2003).

The HMPV M gene has been found to have a molecular mass of 27.6KDa and encodes a 254 amino acid protein that closely resembles the M proteins of other pneumoviruses but only shares 37-38% sequence homology with HRSV (Bastien *et al.*, 2003; van den Hoogen *et al.*, 2002). It has 76-87% homology with the M proteins from AMPV and the ORF is exactly the same size as the other Metapneumoviruses. Within the sub-groups of HMPV, the M gene has 94-100% sequence identity and between the two sub-groups 83-85% homology (Bastien *et al.*, 2003; Biacchesi *et al.*, 2003; Ishiguro *et al.*, 2004; van den Hoogen *et al.*, 2002). The majority of points of variation are conserved and distributed throughout the entire protein and a short region at the N terminal end is conserved between all pneumoviruses.

Two small secondary ORFs in the HMPV M gene at positions 2281 and 2893 have been observed representing 54 and 33 amino acid in length respectively but no start or stop codons have been identified and they share no significant homology with any other secondary ORFs of other pneumoviruses (van den Hoogen *et al.*, 2002).

#### **1.8.3 The surface glycoproteins**

The HMPV and HRSV envelope proteins are F, G and SH glycoproteins which are found embedded within the plasma membrane and expressed on the surface of the virion.

### **1.8.3.1 Attachment (G) protein**

#### **1.8.3.1.1 Structure and processing**

The attachment protein of the pneumoviruses expresses no resemblance to the *Paramyxoviridae* attachment proteins H (hemagglutinin) and HN (hemagglutinin-neuraminidase) (Wertz *et al.*, 1985). It is an anchored type II transmembrane protein that is thought to mediate attachment to the host cell membrane. In HRSV, the protein exists as two forms: a transmembrane protein (G<sub>m</sub>) which is incorporated into virions and as a soluble protein (G<sub>s</sub>) which is secreted by infected cells (Hendricks *et al.*, 1987; Hendricks *et al.*, 1988).

The 289-299 amino acid HRSV G<sub>m</sub> polypeptide is positioned with the N terminus orientated in the cytoplasm and the C terminal exposed extracellularly. A hydrophobic domain in the N terminus (amino acids 38-66) operates as a non-cleaved signal and transmembrane anchor domain which ensures transportation to the ER and subsequent translocation across the membrane (Wertz *et al.*, 1985).

The protein has an estimated molecular weight of 32KDa, however, due to extensive modification with both N- and O-linked glycosylation, the mature protein is converted to 80-90KDa when visualised by polyacrylamide gel electrophoresis. Intermediate species of approximately 45KDa, with the attachment of four high mannose N-linked sugars, have been identified prior to the addition of O-linked sugars in the Golgi compartment (Collins and Mottet, 1992; Wertz *et al.*, 1989). The majority of carbohydrates are attached via O-glycosidic bonds to serine and threonine residues which make up 30.6% of the total amino acid composition (Wertz *et al.*, 1985). Expression of HRSV G in cells defected in protein O glycosylation are only present as 45KDa intermediates (Wertz *et al.*, 1989). Expression of unglycosylated G protein has been detected on the cell surface but in reduced amounts and the presence of carbohydrate side chains appears to be essential for viral infection. The extent of glycosylation is dependent of the host cell and infection of different cell lines may lead to a change in glycosylation profile and a consequent difference in antigenicity of the protein (Garcia-Beato *et al.*, 1996; Melero *et al.*, 1997).

The C terminal ectodomain mainly consists of two highly variable mucin like domains that contain a high content of serine, threonine and proline residues and as a result are extensively glycosylated (Collins and Crowe, 2007). This area is believed to have an extended, unfolded secondary structure (Langedijk *et al.*, 1996; Melero *et al.*, 1997). Interrupting these two structures is a conserved central hydrophobic region (amino acids 164-176) which is devoid of carbohydrate binding sites. Overlapping the conserved domain are four cysteines (residues 173, 176, 182 and 186) which are conserved in all HRSV strains. Disulphide bridges occur between Cys 173 and 186 and between Cys 176 and 182 to form a cysteine noose motif (Johnson *et al.*, 1987b).

G<sub>m</sub> assembles into homoligomers that are proposed to be trimers or tetramers (Escribano-Romero *et al.*, 2004). Within each monomer lies an N terminus comprising a 37 amino acid cytoplasmic region followed by a transmembrane region spanning residues 38 to 66. The first hypervariable region preceding the cysteine cluster is thought to assume a rod-like structure representing the stalk of the protein due to the tightly spaced O-linked sugars. This is followed by the cysteine noose, at the surface of which lies a hydrophobic pocket thought to be involved in the receptor binding (Langedijk *et al.*, 1996; Melero *et al.*, 1997). The second mucin-like region runs anti parallel to the first, back towards to membrane and there has been suggestion the two halves of the C terminal domain interacting to produce an extra fold (Melero *et al.*, 1997).

The secreted form (G<sub>s</sub>) of the HRSV G protein lacks the first 48 amino acids of G<sub>m</sub> as a consequence of initiation of translation at a second in-frame start codon located within the transmembrane domain. The primary translation product enters the exocytic pathway where it is subsequently proteolytically trimmed to remove the remaining transmembrane domain and is subsequently secreted as a heavily glycosylated monomeric soluble protein (Escribano-Romero *et al.*, 2004; Hendricks *et al.*, 1988; Roberts *et al.*, 1994). It is estimated that the soluble form accounts for up to 16-20% of the total G protein synthesised and up to 80% of the G protein released into the medium by 24 hour post-infection (Hendricks *et al.*, 1988).

### 1.8.3.1.2 Function

The HRSV G protein was initially proposed as the attachment protein as antibodies specific for G inhibited the absorption of virus to HeLa cells (Levine *et al.*, 1987). The receptor for HRSV has yet to be properly defined however studies have revealed that the virus can bind to GAGs, on proteoglycans (Hallak *et al.*, 2000a) mainly mediated by the G protein (Techarpornkul *et al.*, 2002). GAG chains are covalently joined to the proteoglycans on the cell surface commonly through serine residues. The net negative charge of the proteoglycans, as a result of high levels of sulphate groups, is the basis for interactions with the positively charged regions of protein ligands (Feldman *et al.*, 1999). Specifically, GAGs containing iduronic acid like heparin sulphate and chondroitin sulphate B have been shown to be important for interaction with HRSV (Hallak *et al.*, 2000b).

Studies with linear peptides have identified a positively charged region (amino acids 184-198) involved in binding heparin (Feldman *et al.*, 1999). However, this heparin binding domain is not the sole determinant of heparin binding since recombinant HRSV lacking that segment of G could still infect *in vitro* in a GAG dependent manner (Teng *et al.*, 2001). Furthermore, studies have shown recombinant HRSV mutants lacking the G protein can replicate in cell culture, although they are highly attenuated *in vivo* (Karron *et al.*, 1997; Techarpornkul *et al.*, 2001). Whilst the G protein is thought to mainly mediate cell attachment, a study where only the F glycoprotein was expressed on the surface revealed the virus could still infect certain cell types in a proteoglycan dependent manner (Techarpornkul *et al.*, 2002) suggesting a role for F protein attachment.

Along side the heparin binding domains, the G glycoprotein of HRSV contains a CX3C motif between amino acids 182-186 (Johnson *et al.*, 1987b). This motif is able to bind to the chemokine fractalkine CX3CR1 receptor present on the surface of cells. The interaction between the G glycoprotein and the CX3CR1 allows modulation of the immune response and inflammation which could potentially slow virus clearance and aid infection (Tripp *et al.*, 2001).

### 1.8.3.1.3 The G glycoprotein of HMPV

Similar to HRSV, the HMPV G protein is a predicted anchored type II transmembrane glycoprotein which is thought to mediate attachment during viral infection (Biacchesi *et al.*, 2003; Biacchesi *et al.*, 2004b; Ishiguro *et al.*, 2004; Peret *et al.*, 2004; Skiadopoulos *et al.*, 2006; van den Hoogen *et al.*, 2002) and whose length varies from 217 to 236 amino acids. This variation in length is partly due to the usage of four different transcription termination codons at nucleotides 652, 658, 685 and 709 and partly due to nucleotide substitutions. These substitutions are mainly situated in the extracellular domain, giving rise to the variation within the G protein and may be a direct result of immunological pressure. Sequence variation is extremely high as a result with 58% nucleotide identity between the sub-groups and a slightly higher percentage identity of 76% between members of the same sub-group. An increased level of divergence at the amino acid level in comparison to nucleotide level is suggestive of a selective pressure for amino acid change by the hosts immune response. It also implies the protein is relatively tolerant of variation which might be due to its proposed extended, unfolded structure (Bastien *et al.*, 2004; Biacchesi *et al.*, 2003; Galiano *et al.*, 2006; Ishiguro *et al.*, 2004; Ludewick *et al.*, 2005; Peret *et al.*, 2004; van den Hoogen *et al.*, 2004).

Recent studies by Thammawat *et al* (2008) have revealed that infectivity and G protein binding can be inhibited by soluble GAGs or by the enzymatic removal of cellular GAGs, indicating that the electrostatic interactions between the positively charged virion proteins and the negatively charged GAGs play a key role in HMPV infectivity. Nonetheless, pre-treatment of cells with soluble G protein could not inhibit infectivity to the level observed with competing GAGs, suggesting that similar to HRSV, interactions of other proteins with cellular receptors may play a role in attachment and infectivity, namely the F protein (Thammawat *et al.*, 2008).

For HMPV, HRSV and AMPV, a small secondary ORF within the G gene can be found however, instead of being within the primary ORF, it follows on from the main ORF and encodes a 68 amino acid protein. Unusually, for HMPV, a third ORF has also been found in the second reading frame overlapping the first two that encodes a 194 amino acid protein and is followed by a fourth encoding a 65 amino acid protein.

Finally, a fifth ORF in the third reading frame has been identified with the potential to encode a 97 amino acid protein. No gene start or gene end sequences have been identified for these ORFs and so it is not known whether they are expressed (van den Hoogen *et al.*, 2002).

HMPV G has a similar hydrophobicity profile but higher threonine / serine and proline residue content compared to the other pneumoviruses especially in the extracellular domain suggesting the protein is a heavily glycosylated mucin like structure (Bastien *et al.*, 2004; Biacchesi *et al.*, 2003; Galiano *et al.*, 2006; Ludewick *et al.*, 2005; Peret *et al.*, 2004; van den Hoogen *et al.*, 2002). Up to five potential N-linked and more than forty O-linked glycosylation sites are responsible for this level of glycosylation and may be important in evading the host immune responses by masking specific epitopes and evading antibody recognition. The predicted molecular mass based on the unmodified polypeptide is 23.7KDa but analysis from western blots have revealed it to be actually between 80 and 100KDa confirming the high level of post-translational N and O-linked glycosylation which has been recognized to occur in the trans-Golgi compartment (Liu *et al.*, 2007).

However, unlike HRSV and AMPV, the G protein lacks the 13-amino acid conserved central domain along with the four conserved cysteine residues that make up the cysteine motif, thought to be associated with protein conformation and signalling, and instead possesses only one conserved cysteine residue in the intracellular domain and a potential second in two of the secondary ORFs (Bastien *et al.*, 2004; Galiano *et al.*, 2006; Ishiguro *et al.*, 2004; Peret *et al.*, 2004; van den Hoogen *et al.*, 2002; van den Hoogen *et al.*, 2004). However, Ishiguro *et al* did identify a partially conserved region (amino acids 92-103) between certain strains thought to be involved in attachment to cellular receptors (Ishiguro *et al.*, 2004). HMPV G also lacks a CX3C fractalkine motif found in the G protein of both HRSV and AMPV-C, involved in mediating attachment and influencing the immune response by chemokine mimicry (Biacchesi *et al.*, 2003; Peret *et al.*, 2004; Tripp *et al.*, 2001). The second start codon in the main ORF which is present in the HRSV G protein and gives rise to a secretory form of the glycoprotein is also absent in HMPV which as a consequence, expresses the transmembrane form only (Biacchesi *et al.*, 2003; Roberts *et al.*, 1994).

Recent investigation by Biacchesi *et al* (2004a) has revealed that the deletion of the G protein alone or in combination with the SH glycoprotein did not inhibit replication in cell culture. However, investigations in hamsters and African green monkeys revealed the mutants to have an attenuated profile *in vivo* compared to wild type virus but are capable of inducing high titres of HMPV-neutralising antibodies and conferred complete protection upon challenge with wild type HMPV (Biacchesi *et al.*, 2005a; Biacchesi *et al.*, 2004b).

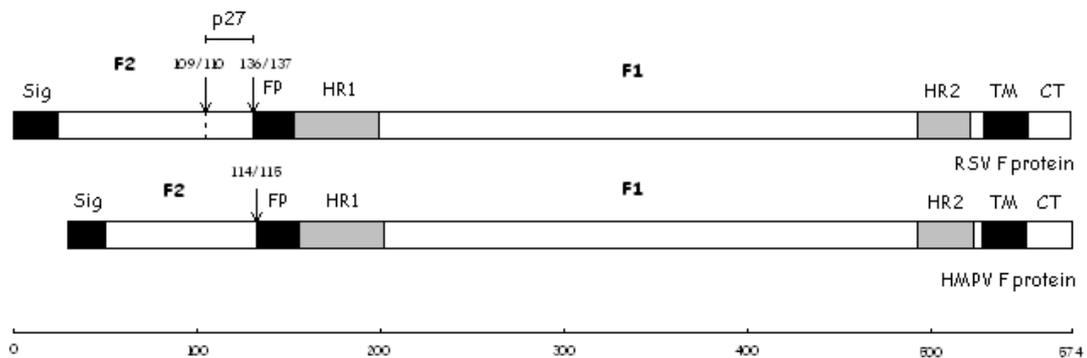
### **1.8.3.2 Fusion (F) protein**

#### **1.8.3.2.1 Structure and processing**

The F glycoprotein of the *Paramyxoviridae* is an anchored type I transmembrane protein that mediates fusion involved in viral entry and syncytium formation. The F gene of HRSV encodes a 574 amino acid protein, which is initially synthesised as inactive precursor, F<sub>0</sub>. The F<sub>0</sub> precursor is translocated to the ER where it is adorned with high-mannose sugar chains which are later modified in the Golgi compartment. This precursor is post-translationally cleaved in the trans-Golgi compartment (Collins and Mottet, 1991; Gonzalez-Reyes *et al.*, 2001) by furin-like proteases during maturation to release a short peptide (p27) producing two disulphide linked polypeptides, F<sub>1</sub> (~50KDa) and F<sub>2</sub> (~20KDa) (Fernie *et al.*, 1985; Gonzalez-Reyes *et al.*, 2001; Zimmer *et al.*, 2001).

The un-cleaved precursor, F<sub>0</sub>, is not detected on the cell surface membrane indicating that cleavage is intracellular. However, as a consequence of incomplete cleavage, cleavage intermediates are detected along with fully processed F proteins in the virions (Gonzalez-Reyes *et al.*, 2001). The two cleavage sites are strictly conserved between all strains of HRSV, site I, between residues 109/110 and site II, between residues 136/137, both of which are preceded by furin-recognition motifs (Melero, 2007; Zimmer *et al.*, 2001). The released 27-mer peptide is thought to facilitate the conversion of the F glycoprotein to its fusogenic form (Zimmer *et al.*, 2001). The expression of unglycosylated mature F proteins on the surface indicates expression is reliant upon cleavage, possibly implicated in transportation to the surface, rather than glycosylation which is thought to help stabilise the protein (Collins and Mottet, 1991).

The F protein of all paramyxovirus have three main hydrophobic regions: one at the N-terminus, removed upon maturation of the glycoprotein, functions as a signal peptide for translocation to the ER during synthesis; the membrane anchor situated at the C-terminus; and the fusion peptide located at the N-terminus of the F<sub>1</sub> chain (Figure 1.3) (Melero, 2007). There are two heptad repeat motifs, HR1 and HR2 situated adjacent to the fusion peptide and transmembrane domain respectively. These are thought to aid the formation and stability of the mature F protein as a homotrimer on the virus and cell surface (Matthews *et al.*, 2000).



**Figure 1.3 Antigenic map of the F glycoproteins of HRSV and HMPV**

Key: Sig, signal peptide; FP, fusion peptide; HR, heptad repeats; TM, transmembrane domain; CT, cytoplasmic tail

### 1.8.3.2.2 Function

The F protein plays an essential role in viral entry, mediating viral / host cell membrane fusion which results in the release of the viral nucleocapsid directly into the cell cytoplasm. Upon attachment of the viral particle to the host cell, the F protein undergoes a conformational change in which the hydrophobic fusion peptide is exposed and leads to insertion into the cell membrane. The trigger mechanism for this change in structure is still unknown (Hernandez *et al.*, 1996).

The F glycoprotein also promotes fusion of nearby adjacent cells resulting in giant multinucleated syncytia formation (Lamb and Parks, 2007; Merz *et al.*, 1980). This is the characteristic cytopathic effect of HRSV and unlike other paramyxoviruses, the F protein can single-handedly cause this phenomenon (Gonzalez-Reyes *et al.*, 2001). Furthermore, the absence of both G and SH glycoproteins in HRSV mutants (Karron *et al.*, 1997) and the production of recombinant HRSV solely expressing F glycoprotein (Techarpornkul *et al.*, 2002) can infect certain cell lines and induce the formation of syncytia indicating the F glycoprotein can serve as an auxiliary attachment protein and induce fusion in the absence of other viral glycoproteins. Nonetheless, HRSV lacking the G glycoprotein is highly attenuated *in vivo* indicating the inefficiency of the F proteins attachment function *in vivo* (Karron *et al.*, 1997; Teng *et al.*, 2001).

The two heptad repeats, HR1 and HR2, play an important role in the fusion process (Chambers *et al.*, 1990). Following insertion of the fusion peptide, the F1 chain is bound simultaneously to the target cell membrane through its N terminus and to the viral membrane via its transmembrane domain (Lamb and Parks, 2007). Conformational changes within the F trimer results in the  $\alpha$ -helical coiled coils of HR1 assembling with the HR2  $\alpha$ -helices to form a 6-helix bundle conformation (Matthews *et al.*, 2000). Situated perpendicular to the viral and host cell membranes, it is postulated that this highly stable structure brings the viral and host cell membranes within close proximity allowing the concomitant fusion of the two membranes by an unexplained mechanism. Lipid mixing of the two membranes allows the formation of a fusion pore connecting the interior of both the viral particle and the host cell cytoplasm (Melero, 2007).

#### **1.8.3.2.3 The F glycoprotein of HMPV**

The 539 amino acid HMPV F glycoprotein resembles that of other *Paramyxoviridae* and has a molecular mass of 58.4KDa (Bastien *et al.*, 2003; Biacchesi *et al.*, 2006; Biacchesi *et al.*, 2004b; Schowalter *et al.*, 2006; van den Hoogen *et al.*, 2002). Similar to HRSV, the F protein is synthesised as an inactive precursor which is cleaved by host cell proteases into two subunits whereupon it remains linked by disulphide bonds. The fusion peptide is situated at the N terminus of the F<sub>1</sub> subunit and is 23

amino acids in length (Biacchesi *et al.*, 2006). However, unlike HRSV (Gonzalez-Reyes *et al.*, 2001), the precursor possesses only one cleavage activation site between residues 114 and 115 (equivalent to site II in HRSV) which does not conform to the consensus furin motif, and is consistent with the data describing the requirement of exogenous trypsin for growth *in vitro* in certain cell lines. Unsurprisingly, growth of HMPV in human bronchial epithelial cells does not require exogenous trypsin for efficient entry and replication (Ingram *et al.*, 2006) and the substitution of serine for proline at the putative cleavage site conferred intracellular cleavability without affecting virulence *in vivo* (Schickli *et al.*, 2005).

As is the case for HRSV, proteolytic cleavage is an essential requirement to process the F protein into a fusogenic form (Schowalter *et al.*, 2006). Groups have recently discovered stimulation of the F protein in certain strains of HMPV requires a low pH, suggesting a role for the lower endosomal pH in entry. Whilst the attachment protein of viruses within the *Paramyxovirinae* subfamily is required for viral attachment and membrane fusion, the G protein of viruses of the *Pneumovirinae* subfamily has been shown to be dispensable for entry into cultured cells (Biacchesi *et al.*, 2006; Biacchesi *et al.*, 2004b; Naylor *et al.*, 2004; Techaarpornkul *et al.*, 2001). Therefore, in the absence of the tight association between the attachment and fusion proteins, the low pH could potentially provide an F protein trigger during HMPV infection (Herfst *et al.*, 2008b; Schowalter *et al.*, 2009; Schowalter *et al.*, 2006).

Similar to HRSV, the F protein of HMPV has been predicted to utilise the formation of hexameric coiled coils of heptad repeats, which has an important role in fusion (Miller *et al.*, 2007). Synthetic peptides derived from the heptad repeat domains have been shown to have a potent inhibitory affect both *in vitro* and *in vivo* (Defrasnes *et al.*, 2008; Miller *et al.*, 2007). Peptides to HR1 were proposed to bind to the metastable prefusion state of the F protein preventing stalk extension and thus arresting the fusion peptide (Miller *et al.*, 2007). The F protein has three potential N-linked glycosylation sites, which may play a role in expression, cleavage and fusion but to what extent is not yet known. Fourteen conserved cysteine residues have been identified with ten positioned closely around the middle of the F1 chain suggesting a role in protein folding (Galiano *et al.*, 2006; van den Hoogen *et al.*, 2002).

The F protein is well conserved between lineages of the same sub-group having 97% nucleotide homology (94% between sub-groups), with small areas of variability and is potentially the main determinant of host tropism (de Graaf *et al.*, 2009). The high nucleotide diversity yet low abundance of amino acid substitutions may be due to structural and functional constraints and as a result plays a major role in the antigenic relatedness between members of the same sub-groups (Boivin *et al.*, 2002; Galiano *et al.*, 2006; Skiadopoulos *et al.*, 2004; van den Hoogen *et al.*, 2004; Winther *et al.*, 2005; Yang *et al.*, 2009). The lack of variability may be responsible for the key role it plays in the induction of neutralising antibodies, which offer protection across both lineages of HMPV (Biacchesi *et al.*, 2004b; Skiadopoulos *et al.*, 2006; Skiadopoulos *et al.*, 2004).

The immunisation with HMPV F protein, in a variety of formats and in several animal model systems, induced protective immunity and in some cases protected against subsequent challenge with both homologous and heterologous strains of HMPV (Cseke *et al.*, 2007; Herfst *et al.*, 2007; Ma *et al.*, 2005; Skiadopoulos *et al.*, 2004; Tang *et al.*, 2005; Williams *et al.*, 2007). Ulbrandt *et al.* (2006) identified a small number of antibodies that cross-neutralised all four HMPV sub-groups both *in vitro* and *in vivo*. The failure of the majority of antibodies to cross-neutralise was suggested to be because neutralising epitopes are preferentially positioned in regions of highest variability as a result of selective pressure (Ulbrandt *et al.*, 2006). Recent work by the same group identified these neutralising regions by the production of MAb - resistant mutants (MARMs). Interestingly, the antibodies that neutralise both *in vitro* and *in vivo* recognise the site on the HMPV F protein that corresponds to the region recognised by the neutralising anti-HRSV monoclonal antibody, Palivizumab. Others MAbs were thought to interact with the pre-fusion conformation of the F protein and prevent the formation of the hairpin loop created by the heptad repeats (The IMPact-RSV Study Group, 1998; Ulbrandt *et al.*, 2008), adding to the speculation that the fusion model is similar to that of HRSV (Miller *et al.*, 2007).

### **1.8.3.3 Small Hydrophobic protein**

The small hydrophobic glycoprotein of HRSV is a short (64-65 amino acids) type II transmembrane protein anchored by a hydrophobic signal/anchor sequence with its

carboxy terminus located extracellularly, whose role in the infectious cycle is not yet known (Collins and Mottet, 1993).

The protein accumulates intracellularly in a variety of forms dependent upon its glycosylation status including an unglycosylated species ( $\text{SH}_0$ ) with a molecular mass of 7.5KDa; a minor glycosylated species of 4.5KDa produced as a consequence of initiation of translation at a second in-frame start codon, an N-linked glycosylated form ( $\text{SH}_g$ ) with a molecular mass of 13-15KDa and a  $\text{SH}_p$  species (molecular mass of 21-60KDa) produced as a consequence of further modification by the addition of polylactosaminoglycans. Each form appears to associate as oligomers that, as determined by chemical cross-linking, form pentamers (Collins and Mottet, 1993).

HRSV recombinants lacking the SH protein gene are fully viable in cell culture (Bukreyev *et al.*, 1997; Karron *et al.*, 1997) and have a slight growth advantage in certain cell lines forming larger plaques. These recombinants however, were attenuated in both mouse (Bukreyev *et al.*, 1997) and chimpanzee (Whitehead *et al.*, 1999) models suggesting the protein plays an essential role in viral pathogenesis. Expression of SH in *E.coli* increased cell permeability to lower molecular weight compounds suggesting the protein may play a role in the formation of membrane channels (Perez *et al.*, 1997). Recombinant HRSV expressing the F and SH proteins only, displayed lower fusion activity and slower viral entry than F glycoprotein alone suggesting the presence of the SH protein had a negative effect on virus fusion in cell culture (Techarpornkul *et al.*, 2001).

#### **1.8.3.3.1 The SH glycoprotein of HMPV**

The SH protein of HMPV is also a type II transmembrane glycoprotein that is inserted into the plasma membrane by a hydrophobic signal-anchor sequence located near its amino terminus (Biacchesi *et al.*, 2004b; Ishiguro *et al.*, 2004; Skiadopoulos *et al.*, 2006). The SH gene located adjacent to the M2 gene is 179 amino acids in length and is the largest SH protein among the pneumovirus subfamily. It is poorly conserved between sub-groups having a nucleotide sequence identity of 69% with a higher percentage identity of 91% between members of the same sub-group (Biacchesi *et al.*, 2004b; Ishiguro *et al.*, 2004; van den Hoogen *et al.*, 2002).

The protein has a high percentage of threonine and serine residues and has a similar hydrophilicity profile to that of the HRSV and AMPV SH protein. Two to four potential motifs for N-linked glycosylation and three to four O-linked glycosylation sites have been observed along with nine to ten cysteine residues being present mainly in the extracellular domain. Nine of these residues are conserved among all strains (Biacchesi *et al.*, 2003; Biacchesi *et al.*, 2004b; Ishiguro *et al.*, 2004; van den Hoogen *et al.*, 2002).

Evidence has been provided that the SH protein exists in multiple forms with varying lengths corresponding to those of HRSV. These forms include SH<sub>0</sub>, which has a molecular mass of 23KDa and is thought to represent the complete unglycosylated SH protein, SH<sub>g1</sub>, which has a molecular mass of 25-30 KDa and is the candidate for the N-linked glycosylated protein and finally SH<sub>g2</sub>, with a molecular mass of 80-220KDa which is thought to be the more extensively glycosylated version (Biacchesi *et al.*, 2003; Ishiguro *et al.*, 2004).

Recent experiments where recombinant mutants have been produced with the deletion of the SH gene suggest that the SH protein has no effect on virus infection or replication *in vitro* and *in vivo* indicating no essential role in attachment and entry. It has also been observed that antibodies to SH have a minor or insignificant role in neutralisation or protection which maybe as a consequence of extensive glycosylation of the extracellular domain of the protein (Biacchesi *et al.*, 2004b; Buchholz *et al.*, 2005; Skiadopoulou *et al.*, 2006). Infection with recombinant HMPV lacking SH expression appeared to enhance secretion of proinflammatory mediators including IL-6 and IL-8 *in vitro* and *in vivo* indicating the SH protein may have a role in mediating NF-κB activation (Bao *et al.*, 2008).

## **1.9 HMPV transmission and clinical manifestations**

HMPV is an important respiratory pathogen and causes both upper and lower respiratory tract infections. HMPV infections are predominantly seen in infants and young children despite the presence of maternally derived antibodies (Bastien *et al.*, 2003; Peiris *et al.*, 2003; Williams *et al.*, 2004) as well as the elderly (Falsey *et al.*,

2003; Stockton *et al.*, 2002; van den Hoogen, 2007) and immunocompromised patients (Boivin *et al.*, 2002; Cane *et al.*, 2003). Severe infection within these groups may result in hospitalisation where mechanical ventilation is required (Falsey *et al.*, 2003; van den Hoogen *et al.*, 2003). It has been estimated that HMPV infection accounts for approximately 5-8% of respiratory tract infections among hospitalised children (Cane *et al.*, 2003; Ijpmma *et al.*, 2004; Peiris *et al.*, 2003) and is thought to be a common cause of nosocomial infections mediated by health care workers in paediatric wards (Mahalingam *et al.*, 2006).

Similar to HRSV, HMPV is contagious and is spread via close contact with infected individuals or by contact with contaminated surfaces. The incubation period from infection to the development of symptoms has been observed to be 4-6 days and infections of HMPV are similar to that of HRSV although less severe. It has been reported that the majority of children over 5 years old were seropositive for HMPV (Boivin *et al.*, 2003; Bosis *et al.*, 2005; Ebihara *et al.*, 2004; van den Hoogen *et al.*, 2001; Williams *et al.*, 2004) and all children were by the age of 10 (Ebihara *et al.*, 2003; Ijpmma *et al.*, 2004). Severe infections are mainly seen in infants less than one year old, possibly due to the small diameter of the bronchioles, which obstruct easily. However, HMPV infections occur slightly later in childhood compared to HRSV. This may reflect an older age at presentation compared to HRSV infections and reflect a more mature immune response or larger airways resulting in an increased ability to clear congestion (Boivin *et al.*, 2003; Ebihara *et al.*, 2004; Klein *et al.*, 2006; van den Hoogen *et al.*, 2001). It has also been postulated that the existence of longer-lasting maternally derived antibodies to HMPV may dampen infection till later on in childhood (Boivin *et al.*, 2003; Mullins *et al.*, 2004).

Upon infection, the virus primarily infects the airway epithelium which results in cell degeneration and necrosis which can be observed in specimens obtained by bronchoalveolar lavage (Kuiken *et al.*, 2004; Vargas *et al.*, 2004). Clinical manifestations can vary from mild respiratory tract infections to bronchiolitis, wheezing as well as pneumonia often accompanied by fever, myalgia and sometimes vomiting with a complication of otitis media in approximately one third of the cases. An influx of peribronchiolar mononuclear cells together with the increase in mucus

secretion and cell debris leads to obstruction of the bronchioles and alveoli (Collins and Crowe, 2007; Vargas *et al.*, 2004).

As for HRSV, clinical outcome is largely based on the age and health of the patient, however, it has been reported that certain HMPV sub-groups may cause more severe disease but for both viruses this still remains controversial (Agapov *et al.*, 2006; Crowe, 2004; Esper *et al.*, 2004; Manoha *et al.*, 2007; Vicente *et al.*, 2006). However, there is evidence suggests that severity of disease is associated with nasopharyngeal viral load where high viral load correlated with disease presentation and not type of HMPV (Bosis *et al.*, 2008).

HMPV infection may induce airway alterations and initiate the development of the atopic state in infants indicating a possible relationship to the onset of childhood asthma. Several studies have revealed an association of HMPV with acute wheezing in older children and the virus could potentially be a stronger trigger for asthma exacerbations than HRSV or influenza (Bosis *et al.*, 2005; Crowe and Williams, 2003; Jartti *et al.*, 2002; Manoha *et al.*, 2007; Mullins *et al.*, 2004; Peiris *et al.*, 2003; van den Hoogen *et al.*, 2003; Williams *et al.*, 2004; Williams *et al.*, 2005a). However, contradictory evidence from an Australian study indicated there was no real association between HMPV and the development of asthma exacerbations (Rawlinson *et al.*, 2003).

Limited data suggest that recurrent infection with HMPV is common, occurring throughout life. This indicates that the host immune response provides transient or incomplete protection and as a consequence allows repeat infection to occur with both homologous (Esper *et al.*, 2004) and heterologous virus strains (Ebihara *et al.*, 2004; Pelletier *et al.*, 2002; van den Hoogen *et al.*, 2004). The young and the elderly can present with both upper and lower respiratory tract disease during the second infection and repeat infections in immunocompromised patients can be just as devastating as the initial illness. Nonetheless the second infection in healthy individuals appears to be milder suggesting neutralising antibodies produced to the initial disease may prevent severe infection even with heterologous strains (Collins and Crowe, 2007; Ebihara *et al.*, 2004; Esper *et al.*, 2004; Pelletier *et al.*, 2002; van den Hoogen *et al.*, 2004).

## 1.10 Animal models

Since the discovery of HMPV in 2001, a number of animal species have been determined to be permissive for HMPV infection (Alvarez *et al.*, 2004a; Darniot *et al.*, 2005; Hamelin *et al.*, 2005; Herd *et al.*, 2006; Kuiken *et al.*, 2004; MacPhail *et al.*, 2004; Skiadopoulos *et al.*, 2004; Williams *et al.*, 2005b; Wyde *et al.*, 2005). These include small animals such as mice, cotton rats, hamsters and primates including chimpanzees, rhesus macaques and African green monkeys. In most of the susceptible animals HMPV replicates to high titres and induces high levels of neutralising antibodies. Research in these models is extremely useful for investigating features of HMPV infection including pathogenesis and anti-viral immunity including the innate and adaptive immune response which will be described later on (Schildgen *et al.*, 2007).

HMPV replication has been shown to be confined to the lungs in both cynomolgus macaques and BALB/c mice where infection was evident in the ciliated epithelium (Alvarez *et al.*, 2004a; Kuiken *et al.*, 2004). Peak viral loads mainly occurred at day 4 (Darniot *et al.*, 2005; Huck *et al.*, 2007; Kolli *et al.*, 2008; Williams *et al.*, 2005b) to day 5 post-infection (Hamelin *et al.*, 2005; MacPhail *et al.*, 2004). Early signs of disease were evident one to two days post-infection, where BALB/c mice exhibited weight loss along with ruffled fur, a tendency to huddle, mucus production and breathing difficulties (Alvarez *et al.*, 2004a; Darniot *et al.*, 2005; Guerrero-Plata *et al.*, 2006; Hamelin *et al.*, 2005; Huck *et al.*, 2007; Kolli *et al.*, 2008). However, experiments involving cotton rats (Williams *et al.*, 2005b), ferrets, hamsters and cynomolgus macaques (MacPhail *et al.*, 2004) experienced no evidence of respiratory illness. MacPhail *et al.* (2004) also discovered that cotton rats were not permissive to HMPV infection which contradicted work by Williams *et al.* (2005) and Wyde *et al.* (2005) where they recovered high levels of infectious virus in both the lungs and nasal mucosa.

Discrepancies in the animal model system could be attributable to the differences arising in the inoculum. HMPV strains from nasopharyngeal secretions have to be isolated in the presence of exogenous trypsin to allow the virus to grow on Rhesus monkey kidney cells (LLC-MK2 or Veros). Before inoculation, these strains were

either passaged in cell culture several times (Darniot *et al.*, 2005; Hamelin *et al.*, 2005; Huck *et al.*, 2007) or filtered (Kolli *et al.*, 2008; Williams *et al.*, 2005b) to establish a high titre stock, allowing for the possibility of certain strains to mutate and adapt to cell culture. Certain groups utilised the extensively passaged prototype strains HMPV/NL/1/00 and HMPV/NL/1/99 (MacPhail *et al.*, 2004) and CAN98-75 (Alvarez *et al.*, 2004a) for their animal model studies, which could have a significant affect on virulence and pathogenesis.

## **1.11 Immunity**

The majority of research to date, studying the immune response to HMPV infection has been restricted to animal models and only limited data is available regarding the immune response in the human host. Both innate and adaptive immunity is believed to play an important role in controlling HMPV replication and persistence. However, primary HMPV infection is thought to elicit a weak innate and adaptive immune response and immunity is incomplete with secondary infections occurring throughout life. Preliminary studies in humans indicate both secretory and serum antibodies are produced in response to HMPV infection. However, the role of these antibodies in protection against reinfection is still unknown (Alvarez and Tripp, 2005; Mahalingam *et al.*, 2006).

### **1.11.1 Innate immunity**

Airway epithelial cells represent the major target of respiratory viruses and upon injury or infection, are able to synthesise and secrete soluble mediators which are important for the recruitment and activation of immune / inflammatory cells. Replication of HMPV induces a variety of cytokines and chemokines, whose expression is dependent on viral replication (Bao *et al.*, 2007).

Recent studies with BALB/c mice have shown that primary HMPV infection elicits a weak innate and humoral immune response, characterised by an early Th1 type response followed by a delayed Th2 type cytokine response with increased IL-10 expression which inhibits the expression of immunoregulatory cytokines (Alvarez and Tripp, 2005; Darniot *et al.*, 2005; Hamelin *et al.*, 2007; Hamelin *et al.*, 2006; Hamelin

*et al.*, 2005; Herd *et al.*, 2006; Panuska *et al.*, 1995). This mirrors observations in HMPV-infected infants (Melendi *et al.*, 2007a) and is postulated to have a role in the association of HMPV and asthma exacerbations.

In infected infants, HMPV elicited significantly lower levels of respiratory cytokines compared to HRSV and levels of cytokines in hospitalised children infected with HMPV compared to infants treated as outpatients were no higher. Despite the similarities between HMPV and HRSV in respiratory tract disease, HMPV may elicit symptoms via independent mechanisms including direct viral damage, Th2 polarisation of the pulmonary immune system and chemokine mediated inflammation, which have all been postulated to play a role in the pathogenesis of HRSV. This suggests a possible common mechanism of illness that is independent of innate inflammation (Laham *et al.*, 2004). Furthermore, the low levels of cytokine induction observed during HMPV infection has also been observed in epithelial cell lines, dendritic cells and animal models (Bao *et al.*, 2007; Guerrero-Plata *et al.*, 2005; Guerrero-Plata *et al.*, 2006).

Other reports have detected significant levels of IL-8 in epithelial cell lines, BALB/c mice and the respiratory secretions of HMPV-infected children with bronchiolitis, the function of which is to recruit and activate neutrophils (Bao *et al.*, 2007; Hamelin *et al.*, 2005; Jartti *et al.*, 2002; Laham *et al.*, 2004). HMPV was also found to be a strong inducer of IL-6, a proinflammatory cytokine (Bao *et al.*, 2007; Douville *et al.*, 2006; Huck *et al.*, 2007; Laham *et al.*, 2004) contradictorily to other findings, where HMPV was found to induce lower levels compared to HRSV (Guerrero-Plata *et al.*, 2005).

The two non-structural proteins of HRSV, NS1 and NS2, are thought to play a major role in blocking initiation of interferon (IFN) production during infection (Bitko *et al.*, 2007). Studies involving recombinant HRSV lacking the NS genes induced high levels of type I interferon in human epithelial cells and macrophages (Spann *et al.*, 2004; Spann *et al.*, 2005). It is known that IFN regulates apoptosis via multiple pathways (Caraglia *et al.*, 2004) and by inhibiting IFN synthesis and response, the NS proteins have been postulated to play a crucial role in suppressing premature apoptosis and augmenting viral replication (Bitko *et al.*, 2007).

Little work has been done in the capacity of HMPV, which lacks NS1 and NS2 proteins, to interfere with IFN responses. However, it is inconceivable that the virus could successfully infect the respiratory tract without countering IFN in some way.

Discrepancies of the immune response to HMPV infection can be possibly assigned to the different properties of isolates, different infection models as well as the time period being evaluated, however the majority of studies have indicated that the immune response to HMPV infection differs significantly from that of HRSV (Douville *et al.*, 2006; Guerrero-Plata *et al.*, 2005; Huck *et al.*, 2007; Laham *et al.*, 2004; Liu *et al.*, 2009; Melendi *et al.*, 2007a).

### **1.11.2 Humoral immunity**

During respiratory virus infection, antibodies play an important role in protection against reinfection or disease. Respiratory virus infection elicits both serum and mucosal IgG, IgA and IgM antibody responses (Mahalingam *et al.*, 2006; Ogra, 2004). Five to ten days following primary infection, an IgM response is induced depending on the age of the patient. This is followed by a specific IgG response which peaks around 20 to 30 days after the onset of symptoms and declines to a low level approximately a year after the primary infection (Ogra, 2004). In subsequent reinfections, a booster effect allows IgG to be detected within five to seven days (Welliver *et al.*, 1980). The IgA response appears slightly later than IgG and IgM.

The predominant antibody found in the lungs is serum IgG, which is induced during infection and mediates protection in the lower respiratory tract (Crowe and Williams, 2003). However, these antibodies are found to be less efficient at conferring protection in the upper respiratory tract, which could be attributed to the inefficient transport of serum antibodies to the mucosa. In the upper respiratory tract, IgA appears to be the major protective antibody and operates independently of the level of serum antibodies (Mills *et al.*, 1971; Prince *et al.*, 1987). The IgA response is short-lived and wanes significantly several months after infection, however, multiple reinfections induce higher levels of secretory antibodies and a more sustained IgA response (Collins and Crowe, 2007; Ogra, 2004; Welliver *et al.*, 1980). Immunity to HRSV infection has been shown to correlate better with high levels of locally sourced IgA antibodies compared to serum antibodies (Prince *et al.*, 1987).

Infants acquire maternal IgG antibodies, primarily IgG1, around the 32<sup>nd</sup> week of gestation and antibody titres of the new born are comparable to that of the maternal level with about a 3-week half life. The passively acquired antibodies are thought to correlate well with protection against lower respiratory tract infections and can be detected up to six months in most infants and perhaps up to one year in some (Collins and Crowe, 2007; Crowe and Williams, 2003).

Antibody responses of young infants to the protective surface glycoproteins of respiratory viruses are significantly lower in frequency and magnitude than that of older children and adults (Crowe and Williams, 2003). Studies in infants with primary HRSV infection demonstrated that most patients aged between 9 and 21 months developed moderate levels of serum and nasal antibodies to both the F and G glycoproteins. However, most infants less than 8 months failed to develop a significant response to these antigens (Murphy *et al.*, 1986a). In addition, infants primarily respond by producing IgG<sub>1</sub> and IgG<sub>3</sub> subclasses and the absence of IgG<sub>2</sub> antibodies to carbohydrates has been postulated to account for the poor response to the highly glycosylated G protein (Crowe and Williams, 2003).

The inability of young infants to elicit a satisfactory antibody response could be a consequence of immunological immaturity or the possibility that the passively acquired maternal derived antibodies suppress the active immune response of the infant. As a result, the incompleteness of protection after natural infection may be partly responsible for susceptibility to the severity of disease in some re-infections in the early years (Collins and Crowe, 2007; Crowe and Williams, 2003; Murphy *et al.*, 1986a).

#### **1.11.2.1 The use of prophylactic anti-RSV antibodies**

There are three major surface glycoproteins present on the membrane surface, F, G and SH, where F and G are thought to be involved in attachment and entry of the virus into the host cell. In HRSV infection, these two surface glycoproteins have been shown to play a major role in host cell immunity and have been shown to be capable of inducing neutralising antibodies. There is evidence to suggest that these neutralising antibodies can confer protection from severe disease and also subsequent reinfection (Taylor *et al.*, 1984; Walsh *et al.*, 1987).

Firstly, in infants, there is a strong correlation between levels of maternal antibodies and protection in the early years of life, when the risk of disease is at its greatest. HRSV infected infants with high levels of maternally derived anti-HRSV antibodies have been shown to have a lower incidence of hospitalisation and reduced severity of disease (Glezen *et al.*, 1981; Ogilvie *et al.*, 1981).

Secondly, studies involving the evaluation of passively transferred serum anti-HRSV antibodies in animal models demonstrated protection in the lower respiratory tract (Prince *et al.*, 1985). Furthermore, therapy with human intravenous immunoglobulin containing high levels of anti-HRSV neutralising polyclonal antibodies (RSV-IVIG) reduce both frequency and duration of hospitalisations and significantly reduced the development of RSV-associated lower respiratory tract infections (Groothuis *et al.*, 1995; Groothuis *et al.*, 1993). RSV-IVIG is now licensed for the prevention of RSV infections in “at risk” infants (Groothuis *et al.*, 1995).

Thirdly, characterisation of the protective human antibody response has led to the development of a partially protective prophylactic monoclonal antibody, palivizumab (Synagis; MedImmune) (The IMPact-RSV Study Group, 1998). Palivizumab is a humanised IgG1 monoclonal antibody that binds to a conserved epitope on the F protein of HRSV and is highly active both *in vitro* and *in vivo* against type A and B clinical HRSV isolates (Beeler and van Wyke Coelingh, 1989; Johnson *et al.*, 1997). Palivizumab was derived from the murine monoclonal antibody, MA b 1129 produced by intranasally immunising BALB/c mice with the A2 strain of HRSV (Beeler and van Wyke Coelingh, 1989). This antibody was then humanised by grafting six complementarity determining regions of the murine monoclonal antibody to a human IgG1 framework. The monoclonal antibody showed broad neutralisation of more than 50 clinical isolates of both HRSV sub-groups and pre-treatment of cotton rats resulted in a 99% reduction of HRSV lung titres (Johnson *et al.*, 1997).

In clinical trials, monthly intramuscular injections of palivizumab (15mg/kg) administered to children with prematurity or bronchopulmonary dysplasia (BPD) reduced the incidence of hospitalisation by 55% compared with a placebo (The IMPact-RSV Study Group, 1998). Palivizumab prophylaxis is currently available to

'at risk' patients and entails the administration of one intramuscular immunisation (15mg/kg) a month for five months surrounding the annual HRSV epidemic period (Deshpande, 2000).

#### **1.11.2.2 Antibody recognition of the F and G glycoproteins of HMPV**

In HMPV infection, there are a number of studies indicating that, in animal models, antibodies correlate with protection. Studies in BALB/c mice showed high serum antibody titres correlated well with a decrease in HMPV lungs titres. Furthermore, passive transfer of HMPV specific antibodies to naïve BALB/c mice provided considerable protection against challenge (Alvarez *et al.*, 2004a; Alvarez and Tripp, 2005).

However, in HMPV, only the F glycoprotein has been found to be highly immunogenic and its highly conserved structure allows the production of cross-neutralising antibodies which confer protection in animal models (Skiadopoulos *et al.*, 2006; Skiadopoulos *et al.*, 2004; Tang *et al.*, 2005). Williams *et al* (2007) used phage display to produce fully humanised monoclonal antibody fragments (Fabs) directed towards the F glycoprotein. Several of these anti-F Fabs neutralised HMPV *in vitro* and effected a virus titre reduction of more than 1000 fold in the lungs of cotton rats suggesting prophylactic potential.

In a recent study, Ulbrandt *et al* (2006) developed a panel of mouse monoclonal antibodies to the F glycoprotein that were cross reactive *in vitro* and *in vivo*. One of these monoclonal antibodies, MAb338, was developed by MedImmune (Hamelin *et al.*, 2008; Ulbrandt *et al.*, 2006). MAb338 was administered intramuscularly into BALB/c mice, which were subsequently intranasally challenged 24 hours later. Five and forty-two days post infection, viral titres were determined and the results revealed lung titres to be significantly lower compared to the control. Histopathological changes were less severe in the pre-immunised mice suggesting a potential role for prophylactic administration (Hamelin *et al.*, 2008).

However, studies of HMPV infection in cynomolgus macaques have shown protection is potentially only transient. Evaluation of vaccine studies, where cynomolgus macaques had been vaccinated three times with HMPV within a ten week period

showed no protective immunity upon HMPV challenge 8 months later despite the presence of neutralising antibodies. Yet challenge 4 to 6 weeks after the primary infection resulted in protection (van den Hoogen *et al.*, 2007).

The variable nature of the G glycoprotein signifies it as a weak neutralising antigen. Studies in hamsters discovered neither the G, or SH glycoproteins induced detectable serum HMPV neutralising antibodies (Skiadopoulos *et al.*, 2006), and immunisations with recombinant viruses expressing either protein were unable to protect the hamsters when challenged. The highly glycosylated structure of the G protein may contribute to the immunogenicity profile and its weak protective nature has been postulated to be attributable to the lack of an essential role in viral entry and replication in model systems (Biacchesi *et al.*, 2005a; Biacchesi *et al.*, 2004b). As a consequence, HMPV, unlike other members of the *Paramyxoviridae*, is thought to have only one major protective antigen.

These results are interesting, given its extensive level of variability, the G glycoprotein must be under extreme immunological pressure to be so diverse. The G glycoprotein of HRSV displays similar high levels of variation thought to be attributed to the selective pressure of circulating antibodies (Cane *et al.*, 1991). Generally MAbs generated towards the G glycoprotein of HRSV are strain or subgroup specific (Taylor, 2007), where recognition of the G protein does not appear to be dependent on its conformational structure (Melero *et al.*, 1997). The limited number of cross-reactive MAbs react with the conserved central region of the G protein, present in all strains of HRSV (Martinez *et al.*, 1997). Despite the lack of neutralising activity, anti-G MAbs have been shown to be effective at protecting mice and cotton rats against HRSV infection (Taylor *et al.*, 1984; Walsh *et al.*, 1984).

Re-infections with heterologous strains of HRSV have been reported as early as two months after primary infection yet repeated infections with homologous strains did not occur until six months after primary infection (Scott *et al.*, 2006). This suggests that whilst primary infection induces largely anti-F cross reactive antibodies, the specific anti-G antibodies provide longer lasting protection (McGill *et al.*, 2004).

### 1.11.3 Cellular immunity

During infections with paramyxoviruses, cell-mediated immunity plays an important role for viral clearance and regulating the immune response, however it is not clear that it confers protection against reinfection and disease (Crowe and Williams, 2003). Alvarez and Tripp have demonstrated that primary HMPV infection in BALB/c mice is associated with a delayed cytotoxic T lymphocyte (CTL) response which coincides with decreased HMPV titre in the lung. Depletion of T cells or natural killer (NK) cells was associated with a significant increase in HMPV titres, suggesting CTL directed responses may have a role in the control of replication and HMPV persistence (Alvarez *et al.*, 2004a; Alvarez and Tripp, 2005). In addition, investigations in which CD4<sup>+</sup> and CD8<sup>+</sup> subsets were depleted individually or together showed they cooperate synergistically in HMPV eradication (Kolli *et al.*, 2008). However, even in the absence of CD4<sup>+</sup> T cells and the consequently impaired generation of neutralising antibodies, mice were still protected from infection, suggesting that CD8<sup>+</sup> subsets potentially could provide adequate protection in secondary infections (Kolli *et al.*, 2008).

The identification of CTL epitopes on HMPV proteins, many of which are conserved across HMPV types, may provide protein targets at the epitope level. Targeting cells expressing these HMPV proteins could prevent viral release and spread and potentially lead to reduced disease severity (Herd *et al.*, 2008). To confirm this observation, Herd *et al* (2006) have recently shown that vaccination of mice with HMPV CTL epitopes reduces both the viral load and HMPV-associated histopathology in the lungs. This vaccination enhances Th1 type cytokine responses, increasing the expression of IL-12 and IFN- $\gamma$ , thus reducing levels of Th2 type cytokines, eradicating the potential of Th2 mediated enhancement of disease. This may be a consideration in the development of future vaccines (Herd *et al.*, 2006). Furthermore, passive transfer of T cell lines against certain epitopes in the M2-1 and N was shown to be protect against challenge (Melendi *et al.*, 2007b).

## 1.12 Candidate vaccines

### 1.12.1 Inactivated vaccines

Trials in the 1960's with the immunisation of infants with formalin-inactivated HRSV (FI-HRSV) vaccine unexpectedly potentiated HRSV disease during subsequent natural infection, leading to a more severe and in some cases fatal disease (Kapikian *et al.*, 1969). Serum analysis revealed that anti-F and anti-G antibodies generated to the vaccine were poorly neutralising being largely non-functional due to the formalin disrupting the antigenic epitopes on the viral proteins (Murphy *et al.*, 1986b).

This phenomenon was reproduced in cotton rats and mice where the immune mechanisms responsible for enhancement of disease have been studied extensively (Taylor, 2007). Immunisations revealed high levels of non-neutralising antibodies that not only failed to protect against infection but led to the deposition of immune complexes and activation of complement (Polack *et al.*, 2002). Further studies revealed live HRSV infection primed mice for a Th1 response, whereas a biased stimulation of Th2 subset of CD4+ T lymphocytes was evident in FI-RSV immunised mice together with an increase Th2 type cytokine expression (Waris *et al.*, 1996). These studies established the animal model for immunopathology which is now being imitated in HMPV studies.

Vaccination of cotton rats with formalin inactivated HMPV conferred almost complete protection upon challenge, yet there was a dramatic increase in lung pathology, resulting interstitial pneumonitis and alveolitis (Boukhvalova *et al.*, 2009; Yim *et al.*, 2007). Similar observations of vaccine enhanced disease were seen after challenging FI-HMPV vaccinated cynomolgus macaques. Serum neutralising antibodies were produced at low levels and immunisation failed to induce protective immunity upon HMPV challenge (de Swart *et al.*, 2007). Enhanced pulmonary disease was observed in BALB/c mice challenged after vaccination with heat inactivated HMPV (potentiated with Freund's complete adjuvant) with a 26% mortality rate yet there was a reduced viral titre and high levels of neutralising antibodies corresponding to protection (Hamelin *et al.*, 2007).

### **1.12.2 Live attenuated vaccines**

Live attenuated vaccines have the advantage of mimicking natural infection without inducing enhanced disease with addition of potentially providing protection against subsequent secondary infections (Herfst and Fouchier, 2008).

Several strategies have been investigated for the development of live attenuated vaccines against HRSV. Extensive passage at suboptimal temperatures allowed the generation of cold-adapted mutants (cp), postulated to be less fit for natural conditions. Growth in the presence of mutagens identified several temperature sensitive mutants (ts), which would be restricted to growth in the upper respiratory tract (Collins and Murphy, 2007). The rationale was that these mutants would be sufficiently attenuated for HRSV-seronegative infants but immunogenic for experienced individuals. However, the chosen vaccine candidates were insufficiently attenuated and caused mild respiratory tract congestion in infants (Wright *et al.*, 2000).

Cp and ts strains of HMPV have been developed by repeated passage at low temperatures. These mutants showed reduced replication in the upper and lower respiratory tract of hamsters nonetheless elicited high levels of neutralising antibodies. Upon challenge following immunisation with these mutants, hamsters were completely protected against challenge with a heterologous strain of HMPV confirming cross-protective immunity (Herfst *et al.*, 2008a). Further investigation of these attenuated strains in cynomolgus macaques, revealed there to be no protection upon challenge 8 weeks after the last immunisation symptomatic of a rapid waning of immunity. Viral neutralising antibodies were only just above the detection limit following the second immunisation suggesting the vaccine was over-attenuated yet an apparent priming of T and B cell responses was enough to shorten the period of virus shedding and accelerate virus clearance (Herfst *et al.*, 2008c).

### **1.12.3 Chimeric vaccines**

The development of a chimeric bovine parainfluenza virus type 3 (PIV3) harbouring the F and hemagglutinin-neuraminidase (HN) genes of PIV3 by Tang *et al.*, 2003, facilitated the expression of the F protein of HMPV. Immunisation of hamsters and African green monkeys induced both PIV3 and HMPV neutralising antibodies that

subsequently protected against PIV3 and HMPV challenge (Tang *et al.*, 2005; Tang *et al.*, 2003).

Hamsters immunised with a recombinant human parainfluenza virus type 1 (PIV1) expressing the F protein of HMPV developed a serum antibody response that neutralised both homologous and heterologous strains. These hamsters were protected from subsequent HMPV challenge from both lineages (as well as PIV1) indicating HMPV F protein is the major antigen mediating cross-protective immunity (Skiadopoulos *et al.*, 2004). Conversely, hamsters intranasally immunised with PIV1 expressing either the G or SH glycoprotein of HMPV did not produce neutralising antibodies even with a booster immunisation. Furthermore, whilst the protective response against HMPV challenge from the G protein was only weak, the response elicited by the SH protein was insignificant (Skiadopoulos *et al.*, 2006), concluding neither the G or SH glycoproteins of HMPV are major neutralising or protective antigens in this system.

Other chimeric vaccines have been generated by replacing the nucleoprotein or the phosphoprotein of HMPV with their counterparts from AMPV-C. These chimeric vaccines induced high levels of neutralising antibodies after intranasal infection. Although there was no significant difference in viral titres in the lungs and nasal turbinates compared to the wild type HMPV. However, there was a 100-1000 fold reduction in replication of the P-chimera in the upper and lower respiratory tract of African green monkeys with the immunogenicity comparable to that of the wild type HMPV (Pham *et al.*, 2005).

#### **1.12.4 Deletion mutants**

Recombinant HMPVs lacking the small hydrophobic, G, or the M2-2 proteins were developed using a reverse genetic system (Biacchesi *et al.*, 2004a; Herfst *et al.*, 2004). These deletion mutants have been reported to efficiently replicate *in vitro* whilst being attenuated in both hamsters and African green monkeys identifying them each as nonessential accessory proteins. Replication of G and M2-2 mutant viruses was reduced 6 and 160 fold respectively in the upper respiratory tract and 3200 and 4000 fold respectively in the lower respiratory tract. Whereas the SH mutant replicated somewhat more efficiently and replication was only slightly lower compared to wild

type HMPV. Upon challenge with wild type HMPV, all deletion mutants were highly protective (Biacchesi *et al.*, 2005a; Biacchesi *et al.*, 2004b; Buchholz *et al.*, 2005). Furthermore, a similar study involving the deletion of M2-2 mirrored the results above and resulted in attenuation in hamsters and conferred protection against challenge with wild type HMPV (Schickli *et al.*, 2008).

#### **1.12.5 Virus replicon particles**

Alpha based replicon particles based on Venezuelan equine encephalitis virus were generated encoding HMPV F or G proteins to test their immunogenicity in both mice and cotton rats. Intranasal inoculation with both constructs elicited significant levels of HMPV-specific IgA antibodies in both the upper and lower respiratory tract which has been shown to be associated with protection against respiratory tract infections. Furthermore, systemic IgG antibodies were detected with HMPV F-specific antibodies possessing neutralising activity. However, the elevated levels of HMPV G-specific antibodies did not contribute to neutralisation or protection against HMPV challenge. Homologous challenge of the vaccinated animals resulted in reduced replication in the lower respiratory tract although HMPV titres were not completely reduced in the nasal turbinates. Surprisingly, despite the inability to produce cross specific neutralising antibodies, vaccinated animals were still protected in the lower respiratory tract when challenged with a heterologous strain. The ability to skew the immune response towards a Th1 type response may have prevented the development of disease potentiation (Mok *et al.*, 2008).

#### **1.12.6 Subunit vaccines**

Due to its highly immunogenic nature, the F glycoprotein has been studied for subunit vaccine development. Antibodies generated to the F protein have been found to be protective both *in vitro* and *in vivo* (Hamelin *et al.*, 2008; Ma *et al.*, 2005; Skiadopoulos *et al.*, 2006; Skiadopoulos *et al.*, 2004; Tang *et al.*, 2005; Ulbrandt *et al.*, 2006; Williams *et al.*, 2007).

Immunisations of Syrian hamsters and cotton rats with soluble F protein resulted in the induction of high levels of virus neutralising antibodies with a more than 1500-fold replication reduction in the lower respiratory tract (Cseke *et al.*, 2007). Generation of two soluble F protein subunit vaccines were evaluated for

immunogenicity, antigenicity and cross-protective efficacy in Syrian golden hamsters. Following two immunisations, high titres of neutralising antibodies were detected and upon challenge with both homologous and heterologous strains, the lower respiratory tract was completely protected against infection (Herfst *et al.*, 2007). Further immunisations in cynomolgus macaques induced HMPV-F specific neutralising antibodies and cellular immune responses but the humoral response waned over time. Challenge 8 weeks following the last immunisation resulted in no lower respiratory tract protection indicating a certain threshold of virus neutralising antibody titres may be needed for protection against subsequent infection (Herfst *et al.*, 2008c).

### **1.13 Vaccinia virus**

The dissection of the humoral response to HRSV infection was greatly facilitated by the individual expression of the viral glycoprotein genes in eukaryotic systems, in which the glycoproteins achieved near-authentic folding and post-translational modifications. Principle among these was the recombinant vaccinia virus system (Moss, 1991). Evaluation of these recombinants in animal models identified the key roles of each viral protein and characterised the correlates of protection (Connors *et al.*, 1991; Olmsted *et al.*, 1988). Expression of HMPV glycoproteins in recombinant vaccinia virus is therefore, an urgent requirement for the progress in the understanding of the human immune response and for the generation of glycoprotein specific monoclonal antibodies which may be of use in prophylaxis.

#### **1.13.1 Classification and structure**

Vaccinia virus belongs to the family of *Poxviridae*, which is split into two subfamilies *Entomopoxvirinae* (insect poxviruses) and *Chordopoxvirinae* (vertebrate poxviruses). Vaccinia virus belongs to the *Chordopoxvirinae* and is classified among the *Orthopoxvirus* genus along with the viruses cowpox and variola virus (Moss, 1996).

Vaccinia virus has a large bricked-shaped virion, which ranges in diameter from 300-400nm. A complex core is surrounded by a lipoprotein envelope containing a single liner double stranded DNA of approximately 200kbp with a hair-pin loop at each end (Moss, 1991; Moss, 1996).

Virus-encoding enzymes including a multi-subunit DNA-dependent RNA polymerase, a transcription factor, capping and methylated enzymes and a poly (A) polymerase are located within the core of the virus and together are involved in the synthesis of translatable mRNA (Moss, 1991).

### **1.13.2 Vaccinia virus as an immunological tool**

A number of expression systems are available for the expression of eukaryotic genes in prokaryotic cells. Despite the ability of bacteria to provide high expression levels, correct folding, proteolytic processing, glycosylation, secretion and subunit assembly may not occur precisely in prokaryotic systems. Therefore eukaryotic genes are best expressed in eukaryotic cells (Fuerst *et al.*, 1986).

Poxviruses have been found to be excellent eukaryotic expression vectors due to the fact that both RNA and DNA synthesis occurs within the cytoplasm instead of the host cell nucleus. This allows the host events to be separated from those of the virus and means potential problems of integration into the host cell genome are not an issue (Hruby, 1990; Moss, 1991). Incorporation of genetic information required to ensure the correct transport of the foreign transcripts from the nucleus to the cytoplasm can also be avoided.

Vaccinia virus, in particular, has a broad host range and has the ability to replicate in many different cell culture lines. Its large genome has the capability of accommodating both large inserts of foreign DNA (>25kbp) and large deletions of viral sequence (>20kbp) and thus has the potential to allow insertion of several different genes into the same genome. Usually a non-essential region of the genome is targeted to allow the virus to replicate independently (Hruby, 1990).

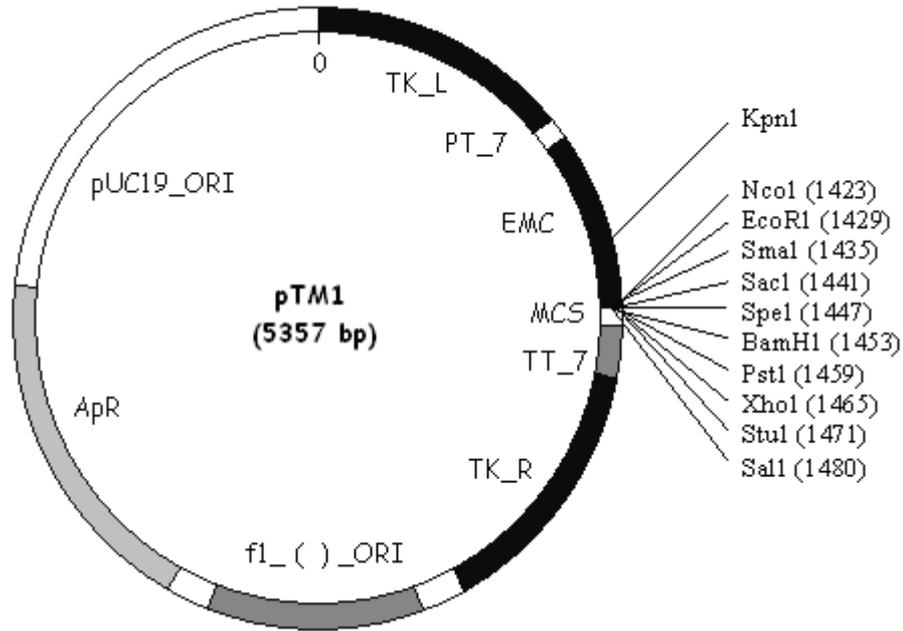
There has been evidence to suggest that foreign genes incorporated into the viral genome can be expected to undergo a variety of post-translational modifications including N and O glycosylation, phosphorylation, myristylation, proteolytic cleavage, polarised membrane and nuclear transport and secretion. The proteins in general are biologically active, transported to the correct cellular compartment and exhibit complete functional activity allowing the protein to have authentic antigenicity (Hruby, 1990; Moss, 1991).

### **1.13.3 Vaccinia virus expression systems**

There are at least two vaccinia virus promoters commonly used in the construction of expression vectors, the 7.5-KDa gene promoter and the 11-KDa promoter. The P7.5 is a constitutive early/late promoter allowing the gene of interest to be expressed in the early and late phase of vaccinia virus infection, whereas the P11 promoter is only expressed as a late gene, after viral replication. Both of these promoters are capable of directing the high-level expression of downstream foreign genes (Hruby, 1990). An alternative, enhancing recombinant protein production, is the bacteriophage T7 promoter. The gene of interest, located downstream of the T7 promoter, is transcribed by the bacteriophage T7 DNA-dependent RNA polymerase, noted for its high-transcriptase activity, stringent promoter specificity and single-subunit structure (Mohamed and Niles, 2004; Moss, 1991).

### **1.13.4 Construction of expression vectors**

Homologous recombination occurs naturally during the replication of poxviruses allowing foreign DNA to be integrated into the viral genome. To exploit this phenomenon for the introduction of foreign genetic material into the virus genome, a shuttle vector must first be constructed where foreign DNA is first integrated into the multiple cloning site (MCS) of a plasmid using restriction endonucleases. Such a shuttle vector is pTM1, in which the MCS is embedded within a region encoding the thymidine kinase gene, which will facilitate efficient recombination between the homologous thymidine kinase (TK) sequence in the vaccinia virus genome. This occurs in about 0.1% of the progeny virions (Moss *et al.*, 1990). The encephalomyocarditis virus independent ribosomal entry site (EMC) is located upstream of the MCS and, in the absence of capping and methylation of the T7 transcripts, is required for efficient cap-independent translation (Elroy-Stein *et al.*, 1989; Fuerst and Moss, 1989). The EMC and MCS are flanked by promoter and terminator elements for the bacteriophage polymerase T7, necessary for expression of the gene of interest when recombination occurs within the vaccinia virus. The plasmid also contains an ampicillin resistance gene that will allow for selection in *Escherichia coli* during cloning, and the bacteriophage f1 single strand DNA origin of replication which allows the plasmid to replicate once inside the bacterial cell (Moss *et al.*, 1990).



TK_L/TK_R	Thymidine kinase gene
PT_7/TT_7	T7 promoter/T7 terminator
EMC	Encephalomyocarditis virus independent ribosomal entry site
MCS	Multiple cloning site
F1_ORI	Bacteriophage f1 origin of replication
pUC19_ORI	pUC19 origin of replication
ApR	Ampicillin resistance gene

**Figure 1.4 Schematic diagram of plasmid, pTM1.**

A number of alternative shuttle vectors can be utilised to aid construction and selection of recombinant viruses, including the related plasmid, pTM3 and pSC11 (Chakrabarti *et al.*, 1985; Mohamed and Niles, 2004). The plasmid, pTM3, contains the *Escherichia coli* guanine phosphoribosyltransferase (gpt) gene that permits selection of recombinant vaccinia virus with mycophenolic acid (Mohamed and Niles, 2004; Moss *et al.*, 1990). In the co-expression vector, pSC11, embedded within the TK sequence, the target gene is under the control of the P7.5 promoter. The presence of a  $\beta$ -galactosidase gene under the control of the vaccinia virus P11 promoter allows additional screening of recombinant plaques with X-gal (Chakrabarti *et al.*, 1985).

In the most widely used format cells are infected with a recombinant vaccinia virus expressing the bacteriophage T7 RNA polymerase, which is under the control of the early/late vaccinia virus P7.5 promoter and so is continuously expressed. The cells are then transfected with the shuttle vector carrying the gene of interest flanked by the T7 promoter and terminator elements (Fuerst *et al.*, 1986).

Homologous recombination occurs between the TK coding region flanking the T7 promoter-regulated target gene in the plasmid and the TK sequence present in the vaccinia virus genome and renders the TK gene inactive (Mackett *et al.*, 1982). TK negative recombinants can be selected using by 5-bromodeoxyuridine (BuDR), which blocks the replication of TK positive virus. This BuDR is a synthetic nucleoside that is an analogue of thymidine. Upon addition to cell culture, the BuDR is phosphorylated by thymidine kinase and incorporated into the nucleotide sequence during DNA replication as a replacement for thymine. However, instead of pairing with adenine as part of the replication process, the conformation of BuDR leads to miss pairing with guanine and consequently alters the nucleotide sequence and thus proteins translated will be non-functional (Earl and Moss, 1991).

Relatively large scale protein expression can be achieved by integrating the T7 promoter-regulated gene into the genome of a second recombinant vaccinia virus. When coinfecting into cells with the recombinant virus expressing the T7 RNA polymerase, high levels of target gene expression can be achieved (Fuerst *et al.*, 1987). Under favourable conditions, levels of recombinant protein made within 24 hours can exceed 10% of the total cell protein (Elroy-Stein *et al.*, 1989). Nonetheless, the co-infection protocol requires accurate determination of virus titres and can be quite costly. However, studies by Fuerst *et al.* (1987) discovered that construction of a recombinant vaccinia virus containing both the T7 RNA polymerase and a T7 promoter-regulated gene seemed to have poor viability. A possible solution is the incorporation of the bacteriophage T7 RNA polymerase gene into a stable cell line. Upon transfection, the target gene under the control of the T7 promoter could be expressed in the presence of the EMC untranslated region. However, protein yields were low and required vaccinia virus infection for high levels of expression (Elroy-Stein and Moss, 1990; Lieber *et al.*, 1989).

## **Chapter 2: Aims**

The aim of this study was to distinguish the immunogenic epitopes of the glycoproteins of HMPV. The initial aims are discussed below.

1. To clone the F, G and SH genes of both subtypes of HMPV into pTM1 in order to produce recombinant vaccinia viruses capable of expressing the individual glycoproteins.
2. To develop a screening assay with these recombinant vaccinia viruses, primarily vaccinia virus expressing the G glycoprotein, that will allow the generation of monoclonal antibodies.
3. To characterise the antibodies generated and assess the ability of the anti-viral glycoprotein antibodies to neutralise

The second part of the project is to develop an animal model that will define the protective immunogens of HMPV. The initial aims are discussed below.

1. To establish the infection model of the four genotypes of HMPV in mice, monitoring the kinetics of viral replication in the lungs and nasal mucosa and lung pathology by histochemical and immunohistochemical methods.
2. Ascertain the protective nature of the G glycoprotein of HMPV by immunisation of BALB/c mice with the vaccinia virus recombinants generated followed by challenge with wild type HMPV.

## **Chapter 3: Materials and methods**

### **3.1 General reagents**

All chemicals were obtained from Sigma (Poole, UK) unless otherwise stated. Distilled water was produced by the Aquatron A4S distillation system (J, Bibby Science Products Ltd., UK). Diethyl pyrocarbonate (DEPC) – treated water for molecular protocols was produced by the addition of 0.1% DEPC to Aquatron purified water, incubated at 37°C overnight and autoclaved.

Reagents, glassware and plastics were sterilised by autoclaving at 121°C, 15lb PSI for 15 minutes.

### **3.2 Immunological reagents**

#### **3.2.1 Primary antibodies**

##### **3.2.1.1 Monoclonal antibodies**

MAb 24 (anti-F glycoprotein), MAb 57 (anti-N protein) and HMPV MAb pool were supplied as hybridoma cell supernatant by Fiona Fenwick (Fenwick *et al.*, 2007).

##### **3.2.1.2 Polyclonal antibodies**

Anti HMPV mouse serum was produced in-house, using strains NCL03-4/145, NCL03-4/174 and NCL03-4/128 – infected human bronchiolar epithelial (16HBE140) cells (Section 3.5.1). All serum collected was absorbed with 16HBE140 cells to remove any cross reactive anti – cell antibody (see Section 3.5.3).

#### **3.2.2 Secondary antibodies**

##### **3.2.2.1 Fluoresceine isothiocyanate (FITC) conjugates**

Sheep Anti-Mouse Fluoresceine isothiocyanate (FITC) conjugated Immunoglobulin (SAM FITC; Novocastra).

##### **3.2.2.2 Horse radish peroxidase (HRP) conjugates**

Polyclonal goat anti-mouse IgG (“NCL-GAMP”) (Novocastra)

Polyclonal goat anti-mouse IgA

In ELISA, both antibodies were used at 1/1000 dilution as determined by checkerboard titration.

### 3.2.3 Isotyping reagents

Control mouse serum (Nordic immunology)

### 3.2.4 Isotype-specific ELISA for immunoglobulins

An ELISA was developed (Section 3.8.4) to identify the generated monoclonal antibodies isotype and quantify the levels of antibody in the hybridoma supernatant when compared with a standard. Goat anti-mouse immunoglobulins specific to IgG1, IgG2a and IgG2b were obtained from sigma and HRSV MAbs were used as positive controls for each isotype (Table 3.1).

**Table 3.1 Dilutions of goat anti-mouse immunoglobulin used to coat ELISA plates**

Goat anti-mouse immunoglobulin	Positive control MAbs
IgG1	1E3
IgG2a	BD51
IgG2b	1A12

A control mouse serum with known quantities of all isotypes was used to calibrate the quantity of antibody in each hybridoma supernatant (Section 3.8.4) (concentrations of isotyped antibodies in Table 3.2).

**Table 3.2 Concentration of each isotype in the standard mouse serum**

Isotype	Concentration (mg/ml)
IgG1	2
IgG2a	8.54
IgG2b	1.25
IgG3	0.754
IgA	2.75
IgM	0.277

### 3.3 Cell culture methods

#### 3.3.1 Cell lines

**Table 3.3 Sources of cell lines used**

<b>Name</b>	<b>Source</b>	<b>Reference</b>
HeLa	Human cervical carcinoma	Clinical Virology unit, RVI
Vero	Monkey kidney	Clinical Virology unit, RVI
16HBE140	Human bronchiole epithelial	Dr Dieter Grunert†
NS-1	Mouse plasmacytoma	Prof, C. R. Madeley‡
TK-143B	Monkey kidney	ECACC*, no 87032605
CV-1	African green monkey kidney fibroblasts	ECACC, no 91112502
L cells	Mouse fibroblasts	Department of Virology, Newcastle University
LLC-MK	Rhesus monkey kidney cells	Clinical Virology unit, RVI

† Cozens et al, 1994

‡ Professor C. R Madeley, Newcastle upon Tyne Health Protection Agency

\* European collection of cell cultures

#### 3.3.2 Cell culture growth media

Medium was prepared under sterile conditions, stored at 4°C and warmed to 37°C before use, unless otherwise stated.

##### 3.3.2.1 Growth medium for HeLa, Vero, LLC-MK and L cells

Eagle minimal essential medium (EMEM), (Cambrex) containing:

10% Foetal calf serum (PAA Labs)

1% 10mg/ml penicillin (Cambrex)

1% 10mg/ml streptomycin (Cambrex)

1% 2mM L-glutamine (Cambrex)

0.02% NaHCO<sub>3</sub> containing phenol red

##### 3.3.2.2 Maintenance medium for HeLa, Vero, LLC-MK2 and L cells

Eagle minimal essential medium (EMEM), (Cambrex) containing:

2% Heat-inactivated foetal calf serum (PAA labs)

1% 10mg/ml penicillin (Cambrex)  
1% 10mg/ml streptomycin (Cambrex)  
1% 2mM L-glutamine (Cambrex)  
0.02% NaHCO<sub>3</sub> containing phenol red

### **3.3.2.3 Growth medium for 16HBE140, TK-143 and CV-1 cells**

EMEM medium as above with 0.1mM Non-essential amino acids (Cambrex)  
TK-143 cells have the addition of 1% 1.5mg/ml 5-Bromo-2-deoxyuridine (BuDR)

### **3.3.2.4 Maintenance medium for 16HBE140, TK-143 and CV-1 cells**

EMEM medium as above with 0.1mM Non-essential amino acids (Cambrex)  
TK-143 cells have the addition of 1% 1.5mg/ml BuDR

### **3.3.2.5 Hank's Balanced Salt Solution (HBSS)**

100ml HBSS (Gibco)  
1% gassed NaHCO<sub>3</sub> containing 4% phenol red (Gibco)

### **3.3.2.6 Growth medium for hybridoma cells**

RPMI-1640 medium (Cambrex) containing:  
2-20% Foetal calf serum (PAA Labs)  
1% 10mg/ml penicillin (Cambrex)  
1% 10mg/ml streptomycin (Cambrex)  
1% 2mM L-glutamine (Cambrex)  
10% BM condimed H1 hybridoma cloning supplement (Roche)

### **3.3.3 Routine cell culture**

Cells were either grown in 4oz sterile glass bottles or plastic tissue culture flasks where confluent cell cultures were split in a ratio of 1:3. The cell medium was removed and the monolayer was washed with 5ml of PBS followed by 5ml of versene containing 1% trypsin and incubated at 37°C for 10 minutes to dissociate the cell monolayer. The cells were then washed with 3ml of growth medium (GM) and the cell suspension was split into 3 bottles; this was carried out approximately every 2-3 days.

### **3.4 Virus and plasmid stocks**

#### **3.4.1 HMPV isolates**

Strains NCL03-4/145, NCL03-4/174 and NCL03-4/128 were all isolated and kindly supplied by Fiona Fenwick and Rosemary McGuckin (Newcastle University, UK)

#### **3.4.2 Vaccinia virus stocks**

Wild type vaccinia virus (strain western reserve [VWT – WS2.1]), was kindly provided by Dr Gail Wertz (Alabama, USA).

VTF7.3 (recombinant vaccinia virus containing the bacteriophage T7 polymerase under the control of the p7.5 promoter) (Fuerst *et al.*, 1987) was kindly provided by Dr Bernard Moss (Laboratory of viral disease, NIAID, Bethesda, Maryland)

Working stocks of wild type vaccinia virus and VTF7.3 was prepared as described in section 3.5.9. Viral titres were established by plaque assay (Section 3.5.11) and infectivity titres ranged from  $7 \times 10^7$  to  $2 \times 10^8$  pfu/ml.

#### **3.4.3 pTM1**

Plasmid pTM1 was kindly supplied by Dr Bernard Moss (Laboratory of viral disease, NIAID, Bethesda, Maryland).

### **3.5 Virological methods**

#### **3.5.1 HMPV inoculation**

Confluent cell cultures of 16HBE140 in 24-well tissue culture plates containing  $1 \times 10^7$  cells were inoculated with 200 $\mu$ l of rapidly thawed virus, diluted 1/10 in ice-cold MM. Plates were centrifuged at 740 x g for 1 hour in a MSE Mistral 3000 centrifuge to aid attachment. A further 800 $\mu$ l of MM was added to all wells and the plates were incubated at 37°C with 5% CO<sub>2</sub>, replacing the medium with fresh MM after 7 days, until 75% of the cells were infected as determined by indirect immunofluorescence (Section 3.5.4). To harvest, the cells were scraped into the medium using a plastic Pasteur pipette, centrifuged at 80 x g for 5 minutes in a bench top centrifuge and the cell pellet resuspended in 3ml of MM. The cells suspension was then aliquoted, snap frozen in liquid nitrogen and stored at -80°C.

### 3.5.2 Production of high titre virus inocula

To obtain high titre virus, HMPV infected cells harvested were subsequently re-infected onto confluent cultures of 16HBE140 cells as described in section 3.5.1. This process was repeated until virus titre increased to sufficient levels established by a fluorescent focus assay (Section 3.5.6). See Table 3.4 for titres of multiple passages of HMPV174, 145 and 128.

**Table 3.4 Yields of multiple passages of HMPV174, 145 and 128 on 16HBE140 cells**

Passage	HMPV strain (titre ffu / ml)		
	174	145	128
4	$5 \times 10^5$	-	$9.6 \times 10^4$
5	$1 \times 10^4$	-	$1.04 \times 10^4$
6	$8.53 \times 10^5$	$5 \times 10^6$	$8.53 \times 10^5$
7	$1.78 \times 10^5$	$3.2 \times 10^5$	$2.42 \times 10^4$
8	$6.13 \times 10^5$	$1.64 \times 10^6$	$7 \times 10^3$
9	$7.2 \times 10^5$	$2.93 \times 10^6$	$5.5 \times 10^5$
10	$7.3 \times 10^5$		$1.2 \times 10^5$
11	$2.6 \times 10^6$		
12	$7 \times 10^6$		

### 3.5.3 Absorption of anti-sera

Four confluent 225cm<sup>3</sup> tissue culture flasks of 16HBE140 cells containing a total of  $2.5 \times 10^8$  cells were washed two times with PBS. Cells were scraped into fresh PBS and pelleted by centrifugation at 700 x g for 20 minutes. The supernatant was removed with a Pasteur in order to remove as much fluid as possible and the pellet was sonicated three times for 30 seconds on ice with 30 second rests in between.

Blood samples collected from immunised mice (see Section 3.15) were centrifuged at 10000 x g for 5 minutes at 4°C in a microcentrifuge. Serum was carefully removed using a Gilson pipette and mixed with the 16HBE140 pellet to resuspend the cells. The cell suspension was placed in a 14ml falcon tube and rotated on a roller at 37°C for 1 hour, which was transferred to 4°C for a further 42 hours. The serum / cell suspension was then centrifuged at 10000 x g for 30 minutes at 4°C in a

microcentrifuge and the serum was carefully removed and stored at 4°C before testing by immunofluorescence.

A further one or two absorptions may have been required to remove all non-specific antibodies.

#### **3.5.4 Indirect fixed cell immunofluorescence**

As described by Gardner and McQuillin, 1980 all antibodies were used, as appropriate, at an optimal dilution determined by checkerboard titration performed by Fiona Fenwick unless otherwise stated.

Cells from infected cultures were fixed onto slides as below. The medium from a well in a HMPV infected 24-well plate (Section 3.5.1) was removed into a sterile tube leaving a residual volume. A small area of cells was scraped into this volume using a Pasteur pipette. 10µl of this cell suspension was placed onto each corresponding spot on a 10-spot multi well slide and the medium replaced in the well to prevent the monolayer from dying out. These were left to dry, fixed in cold acetone for 10 minutes and stored at -20°C until required. When necessary, the slides were defrosted and left to dry. 10µl of primary antibody was added to each spot and the slides were incubated in a moist box for 30 minutes at 37°C. The slides were then washed and left to soak in PBS for 5 minutes before air-drying. 10µl of secondary antibody, 1/50 dilution of SAM-FITC in a 1/1000 dilution of Evan's blue in PBS, was added to each spot. The slides were incubated as above, washed and soaked in PBS for 5 minutes. This was followed by a 1-minute soak in distilled water and the slides were air dried. The spots were observed by ultra violet (UV) light under oil at 50 X magnification using a Nikon Eclipse E400 fluorescence microscope.

#### **3.5.5 Membrane immunofluorescence**

Once HMPV infected 16HBE140 cell cultures had achieved 75% cytopathic effect (CPE) (Section 3.5.1) determined by immunofluorescence (Section 3.5.4), three wells from a 24 well plate of HMPV infected cells and three wells from a negative mock inoculated 16HBE140 control plate were scraped into the medium and pipetted vigorously with a pasteur to break up cell clumps. Each cell suspension was transferred to labelled eppendorfs and microcentrifuged for 2 minutes at 535 x g to

pellet the cells. The cell pellet was washed twice in PBS and finally resuspended in 650µl of PBS. 100µl of serial dilution of mouse serum or mouse monoclonal antibody was added to 100µl of both infected and uninfected cell suspensions and incubated for 1 hour at 37°C in a CO<sub>2</sub> incubator. Following incubation, the cell pellets were washed twice in PBS and resuspended in 50µl of SAM-FITC diluted 1/25 in PBS, and incubated for a further 1 hour as above. After incubation, cell pellets were washed three times as above and resuspended with a drop of PBS. This was transferred to a glass slide, covered with a cover slip and observed under a fluorescent microscope as described in section 3.5.4.

### **3.5.6 Fluorescence focus assay**

After harvesting, all HMPV cultures were titred by a fluorescence focus assay adapted from that used for HRSV (Routledge *et al.*, 1988)

#### **3.5.6.1 Plate preparation and infection**

A 96-well tissue culture plate was seeded with  $3 \times 10^6$  16HBE140 cells/ml in growth medium and incubated overnight in 5% CO<sub>2</sub> at 37°C. Serial ten-fold dilutions of virus sample, up to 10<sup>-7</sup>, were made in cold MM and held on ice. The growth medium on the 96-well plate was discarded and the plate blotted with a sterile paper towel. 25µl of each virus dilution was inoculated in triplicate onto the confluent cells. The plate was then incubated in a moist box for 1 hour at 37°C with 5% CO<sub>2</sub>. The inoculum was discarded and the cells washed with 200µl per well of warm HBSS. 200µl of warm MM was added to the cells, which were then incubated as above for a further 24 hours.

#### **3.5.6.2 Fixing and staining**

At 24 hours post-inoculation, the medium was discarded and the cells were fixed with 75% acetone in PBS for 10 minutes at 4°C. The acetone was discarded and the monolayer air dried, following which, 25µl of HMPV MAb pool was added to each well and the plate incubated for 30 minutes at 37°C. After incubation, the HMPV MAb pool was discarded and the cells were washed twice with PBS soaking for 5 minutes each time. 25µl of a 1/50 dilution of SAM-FITC in PBS was then added to each well and incubated as above. After washing twice with PBS, 200µl of 0.0033%

naphthalene black was added as a counter stain, and the plate was incubated at room temperature for 10 minutes. This was discarded and the plates were washed twice with PBS and once with distilled water and air dried, before viewing under a fluorescence microscope (Nikon Eclipse E400) with a 20x objective lens. The number of fluorescing foci was counted and the titre calculated using the following formula.

(Where f.f.u. means foci-forming units)

$$\text{Virus titre (f.f.u. /ml)} = \text{Average number of foci} \times 40 \times \text{dilution factor}$$

### **3.5.7 Mock neutralisation**

Serial ten-fold dilutions of HMPV up to  $10^{-6}$  were prepared in ice-cold MM. Each dilution was incubated in a round bottomed 96-well tissue culture plate at 37°C with 5% CO<sub>2</sub> for 1 hour. Confluent monolayers of 16HBE140 in a 24-well tissue culture plate were inoculated with 200µl of each virus dilution (four wells per dilution). Plates were centrifuged at 740 x g for 1 hour in a MSE Mistral 3000 centrifuge to aid attachment. A further 800µl of MM was added to all wells and the plates were incubated at 37°C with 5% CO<sub>2</sub>, replacing the medium with fresh MM after 7 days. At 15 days, cells from each well were scraped into medium and transferred to spots on a slide ready for immunofluorescence (Section 3.5.4).

### **3.5.8 Neutralisation**

In a round bottomed 96-well tissue culture plate, 500µl of the appropriate dilution of virus, where approximately 75% of cells exhibited specific antibody staining at 15 days as established from the method above, was incubated with 500µl dilutions of MAbs for 1 hour at 37°C with 5% CO<sub>2</sub>. Alongside this, 500µl of the diluted virus was incubated with 500µl of MM to act as a positive control. After the incubation, 200µl of each virus-antibody mix was transferred to four wells on a 16HBE140 confluent 24-well plate and the plates were centrifuged at 740 x g for 1 hour. A further 800µl of MM was added to all wells and the plates were incubated at 37°C with 5% CO<sub>2</sub>, replacing the medium with fresh MM after 7 days. At 15 days, cells from each well were scraped into medium and transferred to spots on a slide ready for immunofluorescence (Section 3.5.4).

### **3.5.9 Preparation of vaccinia virus stocks**

Wild type and recombinant vaccinia viruses were handled in a contained biohazard category II laboratory. Frozen stocks of virus were thawed rapidly and held on ice, the vials of virus were sonicated 3 x 30 seconds in an ultrasonic bath and returned to ice. To each vial, 1/10th of the sample volume of 0.25% trypsin was added and the vials were incubated at 37°C in a water bath for 30 minutes and then returned to ice.

To prepare virus stocks, 75cm<sup>2</sup> tissue culture flasks containing HeLa cell monolayers were rinsed with warm sterile PBS and 3ml of the virus, diluted 1/10 in MM, was added to each flask. The flasks were incubated at 37°C in 5% CO<sub>2</sub> for 1 hour to allow virus attachment, rocking the flasks every 15 minutes to prevent the cells from drying out.

After the incubation, a further 17ml of MM was added to the flasks and these were returned to the incubator until full CPE was observed. Once this occurred, the cells were harvested into 3ml of MM from a 75cm<sup>2</sup> tissue culture flask and aliquoted into 200µl volumes. The aliquots were freeze/thawed 3 times using solid CO<sub>2</sub>/acetone and stored at -70°C.

### **3.5.10 Passage of inactivated vaccinia virus**

Once inactivated (Section 3.5.18), vaccinia virus was passaged onto veros to ensure inactivation was complete. Frozen stocks of inactivated vaccinia virus were thawed rapidly and held on ice, the vials of virus were sonicated 3 x 30 seconds in an ultrasonic bath and returned to ice. To each vial, 1/10th of the sample volume of 0.25% trypsin was added and the vials were incubated at 37°C in a water bath for 30 minutes and then returned to ice. At the same time, frozen stocks of non-inactivated vaccinia virus was prepared in the same way. Confluent cell cultures of veros in 24-well tissue culture plates were inoculated with 200µl per well of freeze/thawed viruses, diluted 1/10 in ice-cold MM. The plate was incubated at 37°C in 5% CO<sub>2</sub> for 1 hour to allow virus attachment and subsequently topped up with 800µl of MM before being incubated as above for 1 week to monitor infection. This process was repeated once a week for three weeks to ensure the virus was fully inactivated.

### **3.5.11 Vaccinia virus plaque assay**

For virus titration, ten-fold dilutions of virus were made in a total volume of 1ml in MM at 4°C to a dilution of 10<sup>-9</sup>. The growth medium was removed from confluent Vero cells cultured in a 24 well plate and the monolayers were rinsed in warm sterile PBS. 150µl of each virus dilution from 10<sup>-5</sup> to 10<sup>-9</sup> was inoculated in duplicate onto the wells of Vero cells, with 4 wells inoculated with 150µl MM as negative controls. The plate was incubated at 37°C in 5% CO<sub>2</sub> for 1 hour to allow attachment, rocking the plate every 15 minutes to prevent the cells from drying out. After attachment, the virus inoculum was removed and the cells were rinsed with warm sterile PBS. 1ml of MM was added to each well and the plate was incubated as before for 48 hours. After 48 hours, 1ml of 10% formal saline (3.8% formaldehyde in 0.85% sodium chloride) was added to each well and the plate was incubated at room temperature for 10 minutes. The formal saline was then removed and the plate was rinsed under a running tap. Plaques were stained with a drop of 2.5% (w/v) crystal violet for 3 minutes, and then the plate was rinsed under the tap again. Plaques were counted.

Virus titre = Number of plaques x dilution factor x 1/volume of inoculum (pfu/ml)

### **3.5.12 Transient expression of HeLa cells with vaccinia virus VTF7.3 and pTM1 recombinant DNA vectors encoding HMPV glycoproteins**

A 24 well plate was seeded with 3 x 10<sup>6</sup> HeLa cells and incubated overnight at 37°C with 5% CO<sub>2</sub>. The next day, 1ml stock of vaccinia VTF7.3 was rapidly thawed and sonicated for 3 x 30 seconds in an ultrasonic water bath. 100µl of 0.25% trypsin was added to the virus, and the vial was incubated in a 37°C water bath for 30 minutes. The virus was diluted to an MOI of 30 and was held on ice. The HeLa monolayers in 3 wells were washed with warm PBS twice. 500µl of diluted virus was added to the first two wells and 500µl of serum and antibiotic free maintenance medium (SAF MM) was added to the third to act as an uninfected control. The plate was incubated for 30 minutes at 37°C with 5% CO<sub>2</sub>. During incubation, the transfection mix was prepared:

100µl recombinant pTM1 plasmid DNA (10ug)  
5µl Lipofectin reagent (Invitrogen)  
95µl SAF MM

The transfection mix was incubated at room temperature for 15 minutes, and a further 800µl of SAF MM was added. After incubation, the virus was removed from the cells which were washed twice with SAF MM. The transfection mix was added to the first vaccinia virus infected well and 1ml of SAF MM to both the second well of virus infected cells and the uninfected control well and the plate was incubated for 3 hours at 37°C with 5% CO<sub>2</sub>. After incubation, the transfection mix was removed and 1ml of GM was added and the plate was incubated for a further 21 hours.

The cells from each well were scraped into PBS and spot slides were made for indirect immunofluorescence.

### **3.5.13 Recombination of wild type vaccinia virus and pTM1 recombinant DNA vectors encoding HMPV glycoproteins in CV-1 cells**

Three 25cm<sup>2</sup> flasks were seeded from a confluent 75cm<sup>2</sup> tissue culture flask containing 1 x 10<sup>7</sup> CV-1 cells and incubated overnight at 37°C with 5% CO<sub>2</sub>. The next day, 200µl of wild type vaccinia virus was rapidly thawed and sonicated for 3 x 30 seconds in an ultrasonic water bath. 20µl of 0.25% trypsin was added to the virus and the vial was incubated for 30 minutes at 37°C in a water bath. The virus was diluted to give a multiplicity of infection (MOI) of 0.05 pfu per cell in each monolayer in 3ml of SAF MM and was held on ice. The CV-1 monolayers in all flasks were washed twice with warm PBS. 1ml of virus was added to two flasks and 1ml of SAF MM was added to the third as an uninfected cell control. The flasks were then incubated at 37°C with 5% CO<sub>2</sub> for two hours, rocking the flasks every 20 minutes to prevent the cells from over-drying. During the incubation, the transfection mix was prepared:

#### Tube one

3µg Recombinant or control pTM1 DNA from maxiprep  
295µl Serum and antibiotic free MM

### Tube two

30µl Lipofectin® reagent, 1mg/ml (Invitrogen)

270µl Serum and antibiotic free MM

The two tubes were combined and held at room temperature for 15 minutes and a further 2.4ml of SAF MM was added to the mixture. The inoculum was removed from the infected cells and the monolayers were washed twice with warm PBS. 1ml of the transfection mix was added to each of the 3 flasks which were then incubated at 37°C with 5% CO<sub>2</sub> for 30 minutes, rocking the flasks half way through to prevent the cells drying up. After incubation, 9ml of MM containing 5% FCS was added to each flask and the cells were incubated for a further 3.5 hours. The medium was then replaced with 10ml of fresh MM with 5% FCS and incubated as above for 48 hours.

Once full CPE was observed, the cells were scraped into the medium and pooled. The virus/cell suspension was centrifuged at 80 x g for 5 minutes to pellet the cells and resuspended in 1.6ml of SAF MM. This was aliquoted into 200µl volumes, snap-frozen and thawed in solid CO<sub>2</sub>/acetone and stored at -70°C.

### **3.5.14 Plaque purification of recombinant vaccinia virus from transfected CV-1 cells**

#### **Reagents**

##### BuDR stock solution

15mg 5-Bromo-2-deoxyuridine

10ml Water

##### TK- BUdR medium

10ml 10x Eagles minimum essential medium (Cambrex)

10ml Foetal calf serum (PAA Labs)

1ml Non essential amino acids 0.1mM (Cambrex)

1ml 10mg/ml penicillin / streptomycin (Cambrex)

1ml 2mM L-glutamine (Cambrex)

1ml BuDR stock solution

Two 6 well tissue culture plates were seeded with TK-143 cells that had been growing in TK- BUdR medium, and were incubated at 37°C with 5% CO<sub>2</sub> until confluent. A 200µl aliquot of transfected CV-1 cell lysate (from Section 3.5.13) and a 200µl aliquot of wild type vaccinia virus stock were thawed and sonicated for 30 seconds in an ultrasonic water bath. 20µl of 0.25% trypsin was added to both vials and they were incubated for 30 minutes in a 37°C water bath, and then held on ice. Ten fold dilutions of the transfected CV-1 cell lysate to 10<sup>-4</sup> were made in MM containing 8% FCS. The wild type vaccinia was ten fold diluted to 10<sup>-3</sup> in MM. The TK-143 cells in the 6 well tissue culture plates were rinsed with warm PBS, and 500µl of the four dilutions of CV-1 transfected cell lysate, along with the 10<sup>-3</sup> dilution of wild type vaccinia and a “no virus” control well were inoculated in duplicate, into both 6-well plates. The two plates were incubated at 37°C with 5% CO<sub>2</sub> for 2 hours, rocking the plates every 20 minutes to prevent the cells from over drying. After incubation, the inoculum was removed from the plates and the wells in one plate were overlaid with 2% low temperature gelling agarose in EMEM at 37°C containing 1.5µg/ml BuDR (+ BuDR plate). The second plate was overlaid in similar medium but lacking BuDR (No BuDR plate). The plates were incubated at room temperature until the agarose had set, then were transferred to the incubator 37°C with 5% CO<sub>2</sub> for 48 hours. After 48 hours, 2ml of neutral red solution was added to each well and the plate incubated as before until plaques become visible. Well isolated plaques were collected from the + BuDR plate using a pipette tip and were placed in vials containing 200µl of SAF MM. The vials were then snap frozen and thawed three times in solid CO<sub>2</sub> / acetone and stored at -70°C.

### **3.5.15 Production of high titre stocks**

Each plaque pick from section 3.5.14 was rapidly thawed and sonicated 3 x 30 seconds in an ultrasonic water bath. To each vial, 1/10th of the sample volume of 0.25% trypsin was added and the vials were incubated for 30 minutes at 37°C in a water bath.

To prepare virus stocks, a 24-well tissue culture plate containing 1 x 10<sup>7</sup> L cells was rinsed with warm sterile PBS and 100µl of each virus, diluted 1/2 in MM, was added

to each well. The plate was incubated at 37°C in 5% CO<sub>2</sub> for 2 hours to allow virus attachment, rocking the plate every 20 minutes to prevent the cells from drying out.

After the incubation, a further 800µl of MM was added to the wells and the plate was returned to the incubator until full CPE was observed. Once this occurred, the cells in each well were harvested into 400µl of SAF MM and aliquoted into 200µl volumes. The aliquots were freeze/thawed 3 times using solid CO<sub>2</sub>/acetone and stored at -70°C. DNA was extracted from a 200µl aliquot of each plaque pick as described in section 3.5.16, before further passage of an individual clone.

To increase titres, the chosen clone was subsequently passaged from a 24-well plate through to a 225cm<sup>3</sup> tissue culture flask as described above using the volumes stated in Table 3.5.

**Table 3.5 Conditions for passage of vaccinia virus plaque picks**

<b>Tissue culture plastic</b>	<b>Volume of inoculum (ml)</b>	<b>Dilution in MM</b>	<b>Harvested volume (ml)</b>
<b>24 well plate</b>	0.2	1/2	0.4
<b>6 well plate</b>	0.5	1/4	0.8
<b>25cm<sup>3</sup> flask</b>	1	1/10	1
<b>75cm<sup>3</sup> flask</b>	3	1/10	3
<b>225cm<sup>3</sup> flask</b>	5	1/10	9

### **3.5.16 DNA prep**

DNA from a 200µl aliquot of recombinant vaccinia viruses containing the HMPV glycoproteins grown in a 24 well plate (Section 3.5.15) was purified and eluted in 100µl of Buffer AE using a QIAgen DNA purification mini kit (QIAgen) according to the manufacturer's instructions.

These preps were stored at -20°C until required.

### **3.5.17 Coinfection of L cells with recombinant vaccinia virus expressing HMPV G and F glycoproteins and vaccinia virus VTF7.3**

Recombinant vaccinia virus, VTF7.3, was cultured in L cells as described in section 3.5.9 to produce stocks for coinfection. Viral titres were established by plaque assay (Section 3.5.11) and infectivity titres ranged from 7 x 10<sup>7</sup> to 2 x 10<sup>8</sup> pfu/ml.

A 75cm<sup>2</sup> tissue culture flask was seeded with L cells and incubated until confluent at 37°C with 5% CO<sub>2</sub>. A vial of vaccinia VTF7.3 was rapidly thawed and sonicated for 3 x 30 seconds in an ultrasonic water bath. 1/10th of the sample volume of 0.25% trypsin was added and the vial was incubated at 37°C for 30 minutes then held on ice. In parallel, a vial of putative recombinant vaccinia virus clone expressing HMPV glycoproteins (Section 3.5.15) was rapidly thawed, sonicated, trypsinised and incubated as above. Both VTF7.3 and the recombinant vaccinia virus expressing the HMPV glycoprotein was diluted to give an MOI of 3 in a volume of 1.5ml MM containing 5% FCS. The L cells were washed twice with warm PBS and 1.5ml of VTF7.3 was added to the flask. 1.5ml of the recombinant vaccinia virus clone was also added to the flask and rocked to mix. The flask was incubated at 37°C in 5% CO<sub>2</sub> for 2 hours, rocking every 20 minutes to prevent drying. After incubation, the inoculum was removed and replaced with 10ml of MM containing 5% FCS and incubated as above for 22 hours.

To harvest the cells, the medium was removed from each well and the cells were carefully washed with warm PBS. The cells were then scraped into 3ml of PBS and spot slides were made as above and stained for antigen expression by immunofluorescence.

For inactivation (Section 3.5.18), a coinfection of recombinant VTF7.3 and vaccinia virus expressing the HMPV glycoproteins was carried out on a larger scale using 225cm<sup>2</sup> tissue culture flasks. Once the 22-hour incubation period was complete, the medium was removed and the cells were scraped into 9ml of PBS.

### **3.5.18 Inactivation of vaccinia virus expressing HMPV G and F glycoproteins and vaccinia virus VTF7.3**

#### **Reagents**

##### Binary Ethylenimine (BEI) solution

0.175M	Sodium hydroxide in distilled water
2.049g	Binary ethylamine (BEA)

BEI solution was prepared fresh for each inactivation and used immediately. The preparation was incubated at 37°C in a water bath for 30-60 minutes until the pH had dropped from 12.5 to 8.5. Subsequently 3.3ml was added to 96.7ml 2mM EDTA diluted in PBS. Protease inhibitor was added at 1% of the final volume and this solution was sterile filtered with a 2nm filter.

In a bijou, 2.75ml of vaccinia virus infected cell suspension at a concentration of  $3 \times 10^6$  cells/ml was added to 2.75ml of fresh BEI. Along side, 250µl of PBS was added to 250µl of cell suspension to act as a control. The bijoux were then incubated for 24 hours at 37°C, switching the bijoux after two hours to ensure no pockets of virus remained.

After 24 hours, the inactivation process was stopped by the addition of 1M sodium thiosulphate at 10% of the volume of BEI used. The cells were then centrifuged in a bench top centrifuge at 80 x g for 5 minutes. The pelleted cells were resuspended in 5ml of SAF MM, centrifuged again as above to wash the cells and resuspended in a final volume of 2.75ml of SAF MM. This was aliquoted into 200µl volumes, snap-frozen and thawed three times in solid CO<sub>2</sub>/acetone and stored at -80°C.

A vial of each virus was thawed along side the positive control and passed onto a 24 well tissue culture plate containing Vero cells as previously described (Section 3.5.10). The virus was blind passaged once a week for 3 weeks. One week after the third passage, if no CPE developed, the virus was considered inactivated.

## **3.6 Polymerase chain reaction (PCR)**

### **3.6.1 One-way product flow**

Assay set up, RNA extraction and addition, cDNA addition and product visualisation / extraction were all performed in different rooms using separate laboratory coats, pipettes and equipment. RNA, cDNA and amplicand remained in different freezers within the allotted rooms until use in the reaction. A one-way flow system ensured that once the assay mix had been removed from each room throughout the system, it

was not returned to prevent contamination. All plastics and reagents used in each designated area were pre-sterilised and un-opened to prevent contamination.

### **3.6.2 RNA extraction**

#### **3.6.2.1 HMPV**

In an RNase-free environment, total RNA from 2ml of HMPV infected 16HBE140 cells and 2ml of negative control 16HBE140 cells was extracted using a QIAgen RNeasy mini kit. The aliquots were thawed into 600µl of QIAgen lysis buffer containing 0.1% β-Mercaptoethanol and the RNA extraction was carried out according to the manufacturers instructions. The RNA was eluted in 100µl of RNase free water and stored at -80°C.

#### **3.6.2.2 Tissues**

In an RNase-free environment, viral RNA from 140µl of each tissue lysate was extracted using a Viral QIAamp mini kit according to the manufacturers instructions. The RNA was eluted in 40µl of AVE buffer and stored at -20°C.

### **3.6.3 Polymerase chain reaction (PCR) amplifications**

Both RT and PCR reactions were carried out in sterile 0.2ml eppendorf tubes in a PTC-200 Peltier thermal cycler (M.J. Research Inc., USA). All reactions were optimised for magnesium chloride and primer concentration.

### **3.6.4 Reverse transcriptase reaction (RT PCR)**

Reverse transcriptase reactions were carried out in 30µl volumes, 10µl of which was extracted total RNA solution (Table 3.9 and Table 3.11) under the conditions described in Table 3.12. Resulting cDNA was either used immediately or stored at -20°C.

### **3.6.5 PCR**

The reactions were carried out in 50µl volumes containing 5µl of cDNA from the RT reaction (Table 3.7) under the conditions described in Table 3.10. Amplified DNA was either used / visualised immediately or stored at -20°C until needed.

**Table 3.6 Primers used for reverse transcription-polymerase chain reaction amplification from RNA of HMPV128**

<b>Primer set</b>	<b>Primer</b>	<b>Sequence (5'- 3')</b>	<b>Location</b>
1	HMPV 128F F	CGCATATCATGAGTTGGAAAGTGATGATCATCATTTTC	HMPV F (1-29)
	HMPV 128F R	CGCATACTCGAGCTAACTATGTGGTATGAAACCGCC	HMPV F (1620-1597)
2	HMPV 128G F	CGCATACCATGGAAGTAAGAGTGGAGAACATTC	HMPV G (1-25)
	HMPV 128G R	CGCATACTCGAGAACAGTAGATTCACAAAACGGATCCAT	HMPV G (27-1 of L)
3	HMPV 128SH F	CGCATATCATGAAAACATTAGATGTCATAAAAAGTGATG	HMPV SH (1-31)
	HMPV 128SH R	CGCATACTCGAGCGAATGTTCTCCACTCTTACTTC	HMPV SH (26-4 of G)

**Table 3.7 Primers used for reverse transcription-polymerase chain reaction amplification from RNA of HMPV145**

<b>Primer set</b>	<b>Primer</b>	<b>Sequence (5'- 3')</b>	<b>Location</b>
1	HMPV 145G F	CGCATACCATGGAGGTGAAAGTGGAGAACAT	HMPV G (1-23)
	HMPV 145G R	CGCATACTCGAGAACAGTGGATTCATTAAGAGGATCCAT	HMPV G (24-1 of L)

**Table 3.8 Primers used for reverse transcription-polymerase chain reaction amplification from RNA of HMPV174**

<b>Primer set</b>	<b>Primer</b>	<b>Sequence (5' - 3')</b>	<b>Location</b>
1	MF1499F*	GCGGCAATTTTCAGACAACG	HMPV F (626-645)
	MF2175R*	ACATGCTGTTTCGCCTTCAAC	HMPV F (1321-1302)
2	HMPV 174G F <sup>†</sup>	GCGGAATTCCTATGGAGGTGAAAGTGGAGAACAT	HMPV G (1-23)
	HMPV 174G R <sup>†</sup>	GCGGGATCCAACAGTGGATTCATTAAGAGGATCCAT	HMPV G (24-1 of L)
3	HMPV 174G F2	AGCTCACCACCCACAGAATC	HMPV G (155-174)
	HMPV 174G R2	TGTGGACTGTCCGGAGTTGTC	HMPV G (404-385)
4	HMPV 174SH F	CGCATATCATGATAACATTGGATGTCATTA AAAAATGATG	HMPV SH (1-31)
	HMPV 174SH R	CGCATACTCGAGCCATAACTACTTGTCCCATATTTTTG	HMPV SH (4-736 of SH)
5	HMPV 174SH F2	CCACTCTAAACCATGATATAACACAG	HMPV SH (251-276)
	HMPV 174SH R2	CTGTGTTATATCATGGTTTAGAGTGG	HMPV SH (276-251)

Primers were provided by \* Ingram (2006) and <sup>†</sup> Robinson (2007).

**Table 3.9 Optimized reaction conditions for the reverse transcription-polymerase chain reaction amplification from RNA of HMPV**

<b>Reaction (cycling programme)</b>	<b>Reagents (final working concentration)</b>	<b>Volume used</b>
Reverse	Primer (2 $\mu$ M)	1.5 $\mu$ l
Transcription (RT) step	MgCl <sub>2</sub> (5mM)	6 $\mu$ l
	Mixed dNTPs (1mM)	3 $\mu$ l
	RT buffer (1x)	3 $\mu$ l
	RNasin Ribonuclease Inhibitor (30U)	0.75 $\mu$ l
	AMV-RT (8U)	0.8 $\mu$ l
	RNA	10 $\mu$ l
	DEPC treated water	4.95 $\mu$ l
RUTH1 (MF1499F and MF2175R)	All F primers (800nM)	2 $\mu$ l
	MgCl <sub>2</sub> (2mM)	4 $\mu$ l
	Mixed dNTPs (250 $\mu$ M)	1.25 $\mu$ l
	Taq polymerase (1.25U)	0.25 $\mu$ l
	PCR buffer (1x)	5 $\mu$ l
	cDNA	5 $\mu$ l
	DEPC treated water	32.5 $\mu$ l
NHOT (HMPV174GF and HMPV174GR)	All G primers (3.2 $\mu$ M)	8 $\mu$ l
	MgCl <sub>2</sub> (4mM)	4 $\mu$ l
	Mixed dNTPs (250 $\mu$ M)	1.25 $\mu$ l
	Taq polymerase (1.25U)	0.25 $\mu$ l
	PCR buffer (1x)	5 $\mu$ l
	cDNA	5 $\mu$ l
	DEPC treated water	26.5 $\mu$ l
AliT2	All primers (800nM)	2 $\mu$ l
	MgCl <sub>2</sub> (3.5mM)	7 $\mu$ l
	Mixed dNTPs (250 $\mu$ M)	1.25 $\mu$ l
	Taq polymerase (1.25U)	0.25 $\mu$ l
	PCR buffer (1x)	5 $\mu$ l
	cDNA	5 $\mu$ l
	DEPC treated water	29.5 $\mu$ l

RNasin Ribonuclease Inhibitor, Avian Myeloblastosis Virus Reverse Transcriptase (AMV-RT) and 10 x RT buffer were all purchased from Promega. 2' -deoxynucleoside 5' -triphosphates (dNTPs) were supplied by Invitrogen with each nucleoside at a concentration of 10mM in Tris-HCL (pH 7.5). Amplitaq gold DNA polymerase, 10x PCR buffer and magnesium chloride (MgCl<sub>2</sub>) were supplied by Applied Biosystems.

**Table 3.10 Primers used for quantitative PCR**

Primer	Sequence (5' - 3')	Location	Source
N-LN F	CATACAAGCATGCTATATATAAAAGAGTCTC	HMPV N (16-45)	
N-LN R	CCTATTTCTGCAGCATATTTGTAATCAG	HMPV N (178-151)	Maertzdorf <i>et al</i> , 2003
NLN probe	FAM-TGYAATGATGAGGGTGTCACTGCGGTTG-TAMRA	HMPV N (76-103)	

All PCR primers were ordered from MWG Biotech at a scale of 0.2µMol and were purified by HPLC. Probes were ordered from Applied Biosystems at a concentration of 6nmol.

**Table 3.11 Optimized reaction conditions for the real time PCR of RNA from HMPV**

Reaction	Reagents (final working concentration)	Volume used
Reverse Transcription (RT) step for	N-LN F primer (2.75µM)	1.5µl
	MgCl <sub>2</sub> (5mM)	6µl
Quantitative PCR (N-LN F and N-LN R)	Mixed dNTPs (1mM)	3µl
	RT buffer (1x)	3µl
	RNasin Ribonuclease Inhibitor (30U)	0.75µl
	AMV-RT (5U)	0.5µl
	RNA	10µl
	DEPC treated water	5.25µl
Quantitative PCR (N-LN F and N-LN R)	N-LN primers (600nM)	2.4µl
	2x Taqman universal PCR mastermix	15µl
	N-LN probe (200nM)	1µl
	cDNA	6µl
	DEPC treated water	5.6µl

**Table 3.12 Optimized PCR cycle programmes**

<b>Cycle name</b>	<b>Cycle stages and conditions</b>	<b>Number of cycles</b>
RTPCR	Primer extension - 42°C for 30mins	1
	RT enzyme deactivation - 99°C for 5mins	
	Held at 4°C	
RUTH1	Enzyme activation – 94°C for 10mins	35
	Heat Denaturation – 93°C for 1min	
	Primer annealing – 58°C for 1min	
	Primer extension – 72°C for 1min	
Held at 4°C		
NHOT	Enzyme activation – 95°C for 10mins	30
	Heat Denaturation – 93°C for 90secs	
	Primer annealing – 58°C for 90secs	
	Primer extension – 72°C for 90secs	
Held at 4°C		
AlIT 3	Enzyme activation – 95°C for 10mins	30
	Heat Denaturation – 93°C for 90secs	
	Primer annealing – 55°C for 90secs	
	Primer extension – 72°C for 90secs	
Held at 4°C		
QTPCR	Initial denaturation – 95°C for 10mins	45
	95°C for 1min	
	60°C for 15secs	
55°C for 45secs		

### 3.6.6 Reaction product visualisation by agarose gel electrophoresis

#### Preparation of 10x TBE buffer

1 litre DEPC-treated water

900mM Tris base

900mM Boric acid

20mM EDTA

The mixture was adjusted to pH 8.3 with concentrated HCl before autoclaving. Immediately before use 100ul of ethidium bromide (5mg/ml) was added.

### **1% Agarose**

0.5g Agarose (Life Technologies)

50ml 1x TBE buffer

The agarose was heated in the TBE buffer until fully dissolved. The molten gel was mixed with 10µl ethidium bromide (5mg/ml) and transferred directly to a horizontal submarine agarose slab gel mould (Northumbria Biological, UK) to set.

10µl of each reaction product was added to 2µl of 6x sample loading buffer (Promega), and mixed before loading into individual wells of the 1% agarose gel. 12 µl of 100bp DNA or 1KB DNA molecular weight markers (Promega) were also loaded either side. Using a Bio-Rad powerpack (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK), 100V was passed through the gel for 1 hour in 0.5x TBE buffer, and the amplicand band was visualised using a gel ultraviolet transilluminator (Bio-Rad Fluor-S Multimager).

### **3.6.7 Purification of products**

The HMPV gene PCR products were purified by running the amplicand on a 1% agarose gel (Section 3.6.6). The DNA was extracted from the agarose using a QIAquick gel extraction kit (Qiagen) according to the manufacturer's instructions, eluted in 50µl of EB buffer (supplied with the kit) and stored at -20°C.

### **3.6.8 Quantitative PCR (qT-PCR)**

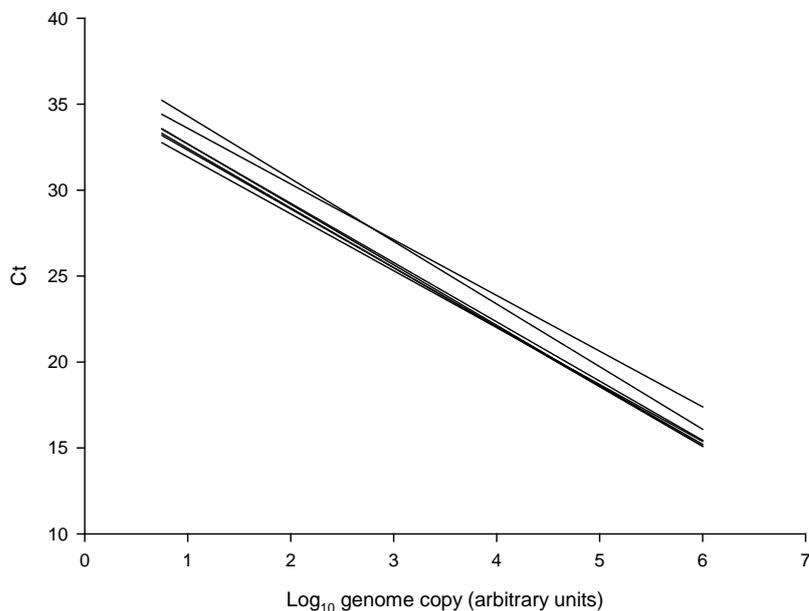
The method for quantitation of HMPV genome was based on the protocol by Dewhurst-Maridor *et al* (2004) with some modifications using the primers and probe designed by Maertzdorf *et al* (2004). The probe, synthesised by Applied Biosystems, which contains a reporter dye (FAM) at the 5' end and a quencher dye (TAMRA) at the 3' end, anneals to the specific cDNA sequence downstream of the N-LN R primer. As the primer is extended by the action of the polymerase during PCR, it cleaves the probe separating the reporter and quencher dyes, resulting in an increased

fluorescence intensity which is proportional to the amount of amplicon being produced in a given sample.

### 3.6.8.1 Standard curve

For quantitation, a standard curve constructed from a sample of known concentration is required. As a RNA standard of known viral RNA content was not available, an arbitrary standard was created using RNA extracted from HMPV174 stock. The amount of viral RNA in a neat sample of this standard was arbitrary assumed to be  $10^6$  units.

RNA, extracted from HMPV174 (infectivity titre  $6.54 \log_{10}$ ), was defrosted and serial 1 in 3 dilutions prepared using DEPC treated water with carrier RNA (made by passing a sample of DEPC water through the QIAamp kit) from neat to 1/177147 ( $10^6$ U to 5.64U). The dilution series was tested in each assay to produce a standard curve (Figure 3.1). At a CT of 25 the mean concentration was  $3.28 \log_{10}$  with a standard deviation of +/- 6.8%.



**Figure 3.1. Standard curves of cycle threshold versus quantity of viral RNA.**

### **3.6.8.2 Reverse transcription reaction**

Reverse transcription reactions were carried out in 48-well PCR plates (scientific specialities inc.) in 30µl volumes. 20µl of mastermix (Table 3.11) was added to the appropriate number of wells followed by 10µl of RNA, mixed by pipetting and the plate sealed using a Microseal 'A' film (M. J. Research, Waltham, U.S.A). The plate was briefly centrifuged and the reaction carried out as described in Table 3.12. Plates were then stored at -20°C until required.

### **3.6.9 PCR amplification**

All quantitative PCR reactions were carried out in 96-well PCR plates (Microplate, Applied Biosystems) in 30µl volumes. 24µl of mastermix (Table 3.11) was added to the required number of wells followed by 6µl of cDNA, tested in triplicate. The cDNA was amplified in an ABI-Prism 7000 Real-Time PCR machine under the described conditions (QT PCR; Table 3.12).

For each reaction, the end point was determined by comparison of the CT value with the standard curve.

## **3.7 Bacterial production of recombinant plasmids**

### **3.7.1 Escherichia coli**

*Escherichia coli* TG1 suppressor strain (K12, D (*lac-pro*), *sup* E, *thi*, *hsd*, 5/F' *traD36*, *proA*<sup>+</sup>*B*<sup>+</sup>, *lacI*<sup>q</sup>, *lacZDM15*) was used as a source of competent cells for transformation experiments.

### **3.7.2 pTM1**

Stocks were prepared by transforming cultures of *E.coli* TG1 with the plasmid pTM1 as described in section 4.1.3. 850µl of the overnight culture (Section 3.7.8) was mixed with 150µl of sterile glycerol, snap frozen in liquid nitrogen and stored at -80°C to be used as a glycerol stock.

### **3.7.3 Reagents**

#### **3.7.3.1 LB medium**

8g Bactotryptone (Oxoid)  
4g Yeast extract (Oxoid)  
8g Sodium chloride  
240µl 5M Sodium hydroxide

Dissolved in 800ml distilled water, autoclaved and stored at 4°C. Before use, 200µl of 50mg/ml ampicillin per 100ml was added.

#### **3.7.3.2 LB agar plates with ampicillin**

8g Bactotryptone (Oxoid)  
4g Yeast extract (Oxoid)  
8g Sodium chloride  
240µl 5M Sodium hydroxide  
6g Bacto™ Agar (Becton Dickinson)

Dissolved in 800ml of distilled water, autoclaved and melted in boiling waterbath and left to cool to approximately 50°C. 200µl of 50mg/ml ampicillin per 100ml was added and poured into sterile Petri dishes (Scientific Laboratory supplies) in the class II cabinet.

#### **3.7.3.3 SOB medium**

8g Bactotryptone (Oxoid)  
2g Yeast extract (Oxoid)  
234mg Sodium chloride  
75mg Potassium chloride

Dissolved in 800ml of distilled water, autoclaved on the same day and stored at 4°C.

On day of use, add

4ml 1M Magnesium chloride  
4ml 1M Magnesium sulphate

#### **3.7.3.4 Transformation buffer**

490mg MES (Mr 195.2) – (2-[*N*-morpholino]ethanesulphonic acid)

Dissolved in 200ml Millipore water and adjusted to pH 6.3

1.85g Potassium chloride

2.22g Manganese II chloride tetrahydrate

367mg Calcium chloride

200mg Hexamine cobalt III chloride

The salts were added to the MES solution, mixed with a stirrer and topped up to 250ml with Millipore water. A 0.2 $\mu$ M filter was rinsed with 2 x 20ml Millipore water and used to filter the transformation buffer into 50ml tubes which were stored at -20°C.

#### **3.7.4 Procedure for making competent bacteria**

5ml of SOB medium was transferred into two sterile universals and subsequently inoculated with *E. coli* TG1 (Section 3.7.1) using a sterile toothpick. These universals were incubated overnight in an orbital shaker at 37°C. The next day, 100 $\mu$ l of TG1 culture was inoculated into a 250ml conical flask containing 60ml of SOB medium preheated to 37°C. The conical flask was incubated as above for 2 hours and the OD was measured at 550nm in a spectrophotometer using SOB medium as a blank. Incubation continued until the OD reached 0.45-0.55.

50ml of the TG1 culture was transferred to a 50ml falcon tube and incubated on ice water for 15 minutes. The culture was centrifuged at 1250 x g for 10 minutes at 4°C and the supernatant was subsequently discarded. The bacteria pellet was resuspended in 5ml of ice cold transformation buffer. A further 15ml of ice cold transformation buffer was added and mixed by inverting the tube. The bacteria were incubated on ice for 10 minutes and then centrifuged at 1250 x g for 10 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in 4ml of ice-cold transformation buffer. 140 $\mu$ l of dimethyl sulfoxide (DMSO) was added and swirled to mix. The solution was incubated on ice for 10 minutes. 140 $\mu$ l of dithiothreitol (DTT)

was added and swirled to mix. This was followed by incubation on ice for 10 minutes and finally addition of 140µl of DMSO was added to the solution and swirled gently.

### 3.7.5 Digestion of purified products for ligation

10µl HMPV cDNA  
 2µl Buffer  
 1µl *Restriction endonuclease1* (New England Biolab)  
 1µl *Restriction endonuclease2* (New England Biolab)  
 6µl DEPC treated water  
 (see Table 3.13 for details)

The reactions were incubated for 3 hours at 37°C in a water bath. Reaction products were then run on a 1% agarose gel as described in section 3.6.6.

**Table 3.13 Restriction digest of HMPV genes with restriction endonucleases**

Gene	Restriction endonucleases for ligation	Buffer number	Restriction endonucleases after transformation	Buffer number
<b>HMPV174</b>	<b>F</b> <i>BamHI</i> and <i>EcoRI</i>	3	<i>BamHI</i> and <i>EcoRI</i>	3
	<b>G</b> <i>BamHI</i> and <i>EcoRI</i>	3	<i>BamHI</i> and <i>EcoRI</i>	3
	<b>SH</b> <i>BspHI</i> and <i>XhoI</i>	4	<i>KpnI</i> and <i>XhoI</i>	1
<b>HMPV128</b>	<b>F</b> <i>BspHI</i> and <i>XhoI</i>	4	<i>KpnI</i> and <i>XhoI</i>	1
	<b>G</b> <i>NcoI</i> and <i>XhoI</i>	2	<i>NcoI</i> and <i>XhoI</i>	2
	<b>SH</b> <i>BspHI</i> and <i>XhoI</i>	4	<i>KpnI</i> and <i>XhoI</i>	1
<b>HMPV145</b>	<b>F</b> <i>BspHI</i> and <i>XhoI</i>	4	<i>KpnI</i> and <i>XhoI</i>	1
	<b>G</b> <i>NcoI</i> and <i>XhoI</i>	2	<i>NcoI</i> and <i>XhoI</i>	2

### 3.7.6 Ligation

Reaction products from section 3.7.5 were purified from enzymes and buffer by running on a 1% agarose gel as described in sections 3.6.6 and 3.6.7.

The digested plasmid pTM1 and HMPV genes were ligated using T4 ligase (2500 Weiss units/ml, Invitrogen) in the reactions described in Table 3.14.

**Table 3.14 Ligation reactions**

<b>Ratio</b>	<b>Plasmid</b>	<b>DNA</b>	<b>5x Gibco ligase</b>	<b>T4 DNA</b>	<b>DEPC treated</b>
<b>Vector : insert</b>	<b>pTM1 (μl)</b>	<b>(μl)</b>	<b>buffer (μl)</b>	<b>ligase (μl)</b>	<b>water (μl)</b>
<b>1:0</b>	1	-	2	1.2	5.8
<b>1:1</b>	1	1	2	1.2	4.8
<b>2:5</b>	4	10	4	2.4	-
<b>1:3</b>	1	3	2	1.2	2.8
<b>1:5</b>	2	10	4	2.4	1.6
<b>0:0</b>	-	-	2	1.2	6.8

Reagents were assembled in sterile eppendorf tubes and incubated in a waterbath at 16°C overnight. The next day, DEPC treated water was added to all tubes to produce a final volume of 100μl and the reactions were stored at -20°C until required.

### **3.7.7 Transformation of competent *E. coli***

2μl, 8μl and 20μl from each ligation reaction (Section 3.7.6) was pipetted into a 14ml Falcon tube and kept on ice. 210μl of competent bacteria preparation (Section 3.7.4) was aliquoted into each of the 18 tubes and incubated on ice for 40 minutes. The bacteria were then heat shocked by placing the tubes in a 42°C waterbath for 90 seconds. The tubes were then transferred to ice for 2 minutes. 800μl of SOB medium was added to each tube and the tubes were incubated for 30 minutes at 37°C. In a class II cabinet, 200μl of each preparation was plated onto a LB + Ampicillin agar plate using a sterile plate spreader. The plates were left to dry then inverted and placed in an incubator at 37°C overnight.

### **3.7.8 Miniprep of recombinant plasmids**

To produce an overnight culture, a sterile loop of each transformed bacteria was streaked onto a LB + Ampicillin agar plate using a sterile plate spreader. Plates were left for approximately 30 minutes at room temperature until the surface was dry. Plates were inverted and incubated at 37°C overnight. Glass universals containing 5ml of LB + ampicillin medium were then inoculated with a single colony from each corresponding plate and left to incubate overnight in the orbital shaker at 37°C.

1.5ml of each of the overnight culture was used in the QIAgen miniprep kit (QIAgen, UK) following manufacturers instructions and eluted in 50µl of EB Buffer (supplied in kit).

### **3.7.9 Maxiprep of recombinant plasmids**

Large scale preparation of plasmid DNA was performed using a QIAgen maxiprep kit (QIAgen) and following the manufacturers instructions. Briefly a 5ml overnight culture of *E. coli* TG1 transformed with the pTM1 plasmids was prepared as stated in section 3.7.8. 100µl of this culture was inoculated into 500ml of LB + ampicillin medium and left to incubate overnight in an orbital shaker at 37°C. The plasmid DNA from this 500ml suspension was eventually eluted in 240µl of EB Buffer (supplied in kit).

Concentration of DNA was determined by diluting the sample in PBS and reading the OD in a spectrophotometer at 260nm according to the following formula:

$$\text{Concentration } (\mu\text{g/ml}) = \text{OD}_{260} \text{ reading} \times \text{dilution factor} \times 50$$

## **3.8 Enzyme Linked Immunosorbent Assay**

### **3.8.1 Preparation of ELISA antigen**

#### **3.8.1.1 HMPV**

Virus infected and uninfected 24-well cultures of 16HBE140 cells were prepared as previously described (Section 3.5.1). After confirmation of 75% CPE determined by immunofluorescence, the medium on both infected and uninfected monolayers was changed to SAFMM and incubated at 37°C in 5% CO<sub>2</sub> for a further 24 hours. Monolayers from 3 plates were then scraped into 1ml of medium, sonicated for 3 x 30 seconds and snap frozen and stored at -80°C.

#### **3.8.1.2 Vaccinia virus**

Coinfected cultures of L cells expressing HMPV glycoproteins and mock coinfecting cultures infected with VTF7.3 alone were prepared in 225cm<sup>3</sup> tissue culture flasks as previously described (Section 3.5.17). After inactivation of the virus with BEI

(Section 3.5.18), the cells were washed and resuspended in 3ml of SAFMM per flask, sonicated 3 x 30 seconds in an ultrasonic waterbath and snap frozen and thawed three times in liquid nitrogen. The antigen was stored at -80°C.

### **3.8.2 Estimation of ELISA antigen protein concentration**

The protein concentration of each ELISA antigen was determined by comparison with known standards using a BIORAD protein assay following the manufacturers instructions.

### **3.8.3 ELISA Reagents**

#### **3.8.3.1 Antigen coating buffer (10X)**

Distilled water to 600ml

9.539g Sodium carbonate

17.64g Sodium bicarbonate

The solution was adjusted to pH 9.6 and autoclaved.

#### **3.8.3.2 Substrate buffer**

Distilled water to 1 litre

5.106g Citric acid

18.426g Di-sodium hydrogen orthophosphate (anhydrous)

The solution was adjusted to pH 5.0 and autoclaved.

#### **3.8.3.3 OPD stock solution**

1.25g O-phenylenediamine dihydrochloride (OPD)

20ml Substrate buffer

Dissolved and aliquoted into 400ml volumes, stored at -20°C

#### **3.8.3.4 OPD substrate solution**

24.6ml Substrate buffer

0.4ml OPD

10µl 30% (v/v) H<sub>2</sub>O<sub>2</sub> solution

This solution was prepared immediately before use

### 3.8.3.5 Stopping solution (3M H<sub>2</sub>SO<sub>4</sub>)

420ml Distilled water

80ml Concentrated sulphuric acid (H<sub>2</sub>SO<sub>4</sub>)

The water was placed on ice and the acid added slowly with mixing at regular intervals.

### 3.8.3.6 Washing buffer (10X PBST/PBSTx)

Distilled water to 2 litres

160g Sodium chloride

4g Potassium di-hydrogen orthophosphate

23g Di-sodium hydrogen orthophosphate (anhydrous)

4g Potassium chloride

10ml Tween 20 (PBS/T or 20ml Triton X-100 (PBS/Tx)

The solution was adjusted to pH 7.4 and autoclaved

### 3.8.4 Isotype

Nunc Maxisorb immuno-plates were coated with 50µl per well of goat anti-mouse isotype immunoglobulins at the dilutions stated in Table 3.15 overnight at 4°C.

**Table 3.15 Dilutions of goat anti-mouse immunoglobulin used to coat ELISA plates**

<b>Goat anti-mouse immunoglobulin</b>	<b>Dilution in coating buffer</b>	<b>Positive control</b>
IgG1	1/800	1E3
IgG2a	1/12800	BD51
IgG2b	1/12800	1A12

The next day, the wells were washed with PBST, three times for 3 minutes, blotting the plates on paper towels between washes. Standard mouse serum, positive control MAbs and MAbs to be tested were serially diluted 1 in 4 from 1/256 to 1/ 1048576 in PBST containing 10% heat-inactivated foetal calf serum (PTF) and 25µl per well of each dilution added to the plates. Plates were incubated at 37°C for 90 minutes in a moist box (sealed box containing a wet paper towel) before washing as above. Secondary antibody, Goat anti-mouse peroxidase-conjugated immunoglobulin

(GAMP IgG), was diluted 1/1000 in PTF and 25µl was added to all wells, which were incubated for 1 hour at 37°C. After further washing, 50µl of substrate buffer was added to each well and incubated for 30 minutes at 37°C, after which the reaction was stopped with the addition of 50µl of stopping solution.

The ELISA plate was read with an MRX II Microplate reader (Dynex Technologies, UK) using revelation software version 4.02 at an optical density of 492nm.

### **3.8.5 Antigen capture ELISA**

The wells of a flat bottomed 96 well plate (Nunc Maxisorb immuno-plate) were coated with 50µl of either HMPV infected or uninfected ELISA antigen (Section 3.8.1.1) (diluted to 24µg/ml in antigen coating buffer) overnight at 4°C. The next day, the wells were washed with PBST, three times for 3 minutes. 25µl per well of primary antibody, diluted in PTF, was added to the plate and incubated at 37°C for 90 minutes in a moist box before washing as above. Secondary antibody, diluted 1/1000 in PTF, was added at 25µl per well and the plate was incubated for 1 hour at 37°C. The rest of the ELISA was performed as described in section 3.8.4.

Antigen concentration was determined by chequerboard titration.

### **3.8.6 Lectin capture ELISA**

The wells of a flat bottomed 96 well plate were coated with 50µl of Concanavalin A at a concentration of 50µg/ml in antigen coating buffer and incubated overnight at 4°C. The next day, the wells of the plate were washed with PBSTx three times for 3 minutes. Inactivated recombinant HMPV antigen or VTF7.3 negative antigen (Section 3.8.1.2) was diluted in PBSTx to a concentration of 23.7µg/ml and 50µl per well added to the plates. Plates were incubated in a moist box for 2 hours at 37°C before washing and blotting as before. Primary antibody was diluted in PTF and 25µl added to each well. Plates were incubated for 90 minutes as before, followed by washing 3 x 3 minutes with PBSTx. The rest of the ELISA was performed as described in section 3.8.4.

Antigen concentration was determined by chequerboard titration.

### **3.8.7 Immobilised immunoglobulin ELISA**

The wells of a 96 well ELISA plate were coated with 50µl of primary antibody diluted from 1/50 to 1/204800 in antigen coating buffer and incubated overnight at 4°C. The next day, the wells of the plate were washed with PBST three times for 3 minutes, blotting in between. Secondary antibody, Goat anti-mouse peroxidase-conjugated immunoglobulin (GAMP IgA), was diluted 1/1000 in PTF and 25µl was added to all wells, which were incubated for 1 hour at 37°C. The rest of the ELISA was performed as described in section 3.8.4.

## **3.9 Immunisation of mice**

### **3.9.1 Mouse stocks**

Female Balb/c mice were ordered from Charles River Laboratories, UK at 6-8 weeks old, and inoculations begun 1 week after arrival. The animals were maintained by the Comparative Biology Centre, Newcastle University.

### **3.9.2 Isofluorane anaesthetic**

Mice were anaesthetised using an isofluorane circuit. Isofluorane initially flowed into the anaesthetic induction chamber (Alfred Cox Ltd) at concentration of 5% with oxygen. Once the chamber was filled, the flow rate was reduced to 2% with waste anaesthetic gases scavenged by a 'fluo-vac' scavenger unit (International Market Supplies). Mice were placed into the chamber, one at a time, until they lost consciousness. Animals were then removed from the chamber and the intranasal inoculations administered (Section 3.10.1) before recovery (Waynforth and Flecknell, 1992).

### **3.9.3 Preparation of antigen in Freund's adjuvant**

Equal volumes of antigen and Freund's adjuvant were drawn into two 2ml syringes. The syringes were joined with a hub, and the mixture was emulsified by repeat plunging of the syringes until a thick paste formed. PBS-tween 20 was subsequently added to the paste at 2 x the final volume using the same technique as above.

### **3.10 Methods of inoculation**

#### **3.10.1 Intranasal inoculations**

Anaesthetised mice (Section 3.9.2) were removed from the chamber and held in a category two hood where 50µl of antigen preparation (live antigen or antigen with a cholera toxin adjuvant) was instilled into the nostrils using a 0.5ml 'LO-DOSE' insulin syringe / needle (Becton Dickinson). The mouth was pushed closed to allow the preparation to be taken up into the lungs.

#### **3.10.2 Subcutaneous inoculations**

Mice were handled by the scruff of the neck and placed onto a bench for ease of handling. Antigen preparation in Freund's adjuvant (complete / incomplete) was then injected into four sites in the neck at 50µl per site using a 1ml syringe and 25G needle.

#### **3.10.3 Intraperitoneal inoculations**

Mice were handled by the scruff of the neck and turned over to expose the peritoneum. 20µl of antigen preparation without adjuvant was inoculated into a posterior quadrant of the abdomen using a 1ml syringe and 25G needle.

### **3.11 Production of hybridomas**

#### **3.11.1 Immunisation schedule**

Day 1	Intranasal inoculation of antigen 50µl / mouse
Day 14	Injection of antigen in Freund's complete adjuvant (Sigma) 20µg / mouse at four sites subcutaneously in the neck
Day 28	Injection of antigen in Freund's incomplete adjuvant (Sigma) 20µg / mouse at four sites subcutaneously in the neck
Day 35	Test bleed

4 days pre-fusion, for the generation of hybridomas, the mice were inoculated by intraperitoneal injection of antigen with no adjuvant, 20µg / mouse in 20µl

### **3.11.2 HAT medium**

HAT medium supplement ( $5 \times 10^{-3}$ M hypoxanthine,  $2 \times 10^{-5}$ M aminopterin,  $8 \times 10^{-4}$ M thymidine) was diluted in 10ml of sterile water. 4.5ml of this HAT solution was added to 10ml of BM Condimed H1 hybridoma cloning supplement (Roche) and made up to 50ml with RPMI-1640 containing 20% foetal calf serum (R20). This HAT / Condimed / R20 solution was sterile filtered into 50ml of R20 and warmed to 37°C.

### **3.12 Fusion technique for the production of hybridomas**

NS-1 cells were cultivated in RPMI containing 10% foetal calf serum and sub cultured at a ratio of 1:2 every day in 75cm<sup>3</sup> tissue culture flasks. Immune mice were sacrificed by cardiac puncture, and the spleen aseptically removed. Splenocytes were dissociated using two curve end forceps by teasing the cells from the spleen into 10ml of R20, and drawing the splenocytes through a 25G needle several times with a 10ml syringe. Both NS-1 cells and splenocytes were counted using a haemocytometer so that the splenocytes were added to the NS-1 cells at a ratio of 8:1. The cell suspension was centrifuged at 1000rpm for 5 minutes, where after nearly all the supernatant was removed and the cells resuspended in the remainder. 1 ml of Polyethylene glycol 1500 (PEG-1500, Roche) was added to the cell mix slowly over 1 minute, gently rotating the tube and keeping the cells warm by immersing the bottom of the centrifuge tube in a beaker of water at 37°C. 1 ml of R20 was then added slowly over a minute, while gently agitating the mixture. 20ml of R20 was then slowly added to the cell suspension over 4 minutes and the cells were poured into 200ml of HAT medium. The cells were then plated out onto 11 x 96 well plates, 200µl per well and incubated for 10 days at 37°C with 5% CO<sub>2</sub>. These plates were screened on inactivated recombinant vaccinia virus antigens (see Section 3.13)

### **3.13 Screening fusions**

#### **3.13.1 Concanavalin A ELISA**

For each hybridoma plate one maxisorb 96-well ELISA plates was coated with 50µl Concanavalin A (ConA) as described in section 3.8.6 and incubated overnight at 4°C.

The next day the wells were washed 3 times with PBSTx and the plates coated with either recombinant HMPV virus antigen or VTF7.3 negative control antigen (Section 3.8.6) as appropriate. The plates were incubated in a moist box at 37°C for 2 hours before washing as described above. Using a transplator (Corning), hybridoma supernatant from each fusion plate was transferred to each corresponding ELISA plate then incubated for 90 minutes as above. The ELISA continued as described in section 3.8.4.

### **3.13.2 Rescreening of HMPV antigen**

Hybridomas were rescreened on HMPV174 infected and uninfected 16HBE140 cells as described in section 3.8.5 transferring the hybridoma supernatants as described in section 3.13.1.

## **3.14 Cloning hybridoma cells**

### **3.14.1 Preparation of spleen feeders**

12 mice were supplied by Charles River (UK) Laboratories. On the day of arrival, each mouse was sacrificed by cervical dislocation and their spleen removed under aseptic conditions. Splenocytes were dissociated using two curve end forceps by teasing the cells from the spleen into 10ml of RPMI-1640, and drawing the splenocytes through a 25G needle several times with a 10ml syringe. The splenocytes were counted using a haemocytometer and frozen as  $1.2 \times 10^8$  cells / vial, enough to seed two plates.

### **3.14.2 Cloning**

A 96-well plate was seeded with  $3 \times 10^6$  / ml of spleen feeders at 200µl per well. Each chosen hybridoma was harvested from the primary well and counted using a haemocytometer and diluted to 1000 cells in 2ml of HAT medium. 100µl was added to the first 2 columns of the feeder plate and subsequently two fold diluted down the plate as to isolate individual cells. The plates were incubated at 37°C with 5% CO<sub>2</sub> for a week or more and screened again on either HMPV or vaccinia virus (Section 3.13).

### **3.15 Extraction of blood**

Blood samples were taken from the lateral tail vein with the mouse restrained in a purpose built restraining device. A maximum of 10% of the circulating volume was taken on a single occasion.

Mice were also bled by cardiac puncture when requiring a larger volume and where maximum amounts needed to be retrieved, mice were anaesthetised by intraperitoneal injection of Fentanyl / fluanisone and midazolam (2.7 ml/kg) prior to cardiac puncture (Flecknell, 2009).

### **3.16 Extraction of tissues**

Mice were sacrificed by cervical dislocation. Using aseptic techniques, the lungs and nasal mucosa were removed, weighed and placed into 1ml of MM on ice. One lobe of the right lung was clamped with a pair of Spencer wells artery clamps and inflated with neutral buffered formalin to be later used for immunohistochemistry (Section 3.19). The rest of the tissues were homogenised using Griffith's grinders and suspension was centrifuged at 300 x g for 5 minutes at 4°C. The supernatant was carefully removed and kept on ice to be used for quantitative PCR (Section 3.6.8) and infectivity assay (Section 3.17).

### **3.17 Infectivity assay**

A 96-well tissue culture plate was seeded with  $3 \times 10^6$  16HBE140 cells/ml in growth medium and incubated overnight in 5% CO<sub>2</sub> at 37°C.

The next day, the growth medium was discarded and 25µl of each homogenate was inoculated in triplicate onto the confluent cells. The plate was then incubated in a moist box for 90 minutes at 37°C in 5% CO<sub>2</sub>. The inoculum was discarded and the cells washed with 200µl per well of warm PBS. The rest of the assay was performed as described in section 3.5.6.

Virus titre (f.f.u / g) = Average no. of foci x 40 x dilution factor x 1/ (weight of lung in g) x 2

## 3.18 Challenge

### 3.18.1 Immunisation schedule

Day 1	Injection of antigen in Freund's complete adjuvant (Sigma) 20µg / mouse at four sites subcutaneously in the neck (Section 3.10.2)
Day 14	Injection of antigen in Freund's incomplete adjuvant (Sigma) 20µg / mouse at four sites subcutaneously in the neck
Day 28	Injection of antigen in Freund's incomplete adjuvant (Sigma) 20µg / mouse at four sites subcutaneously in the neck
Day 42	Injection of antigen in Freund's incomplete adjuvant (Sigma) 20µg / mouse at four sites subcutaneously in the neck
Day 366	Intranasal inoculation of 10µl of antigen containing 20ng Cholera toxin and 2µg Heat-labile Enterotoxin, B subunit from <i>E. Coli</i> (LTB) under isofluorane anaesthetic (Section 3.10.1)
Day 380	Intranasal inoculation of 10µl of antigen containing 20ng Cholera toxin and 2µg Heat-labile Enterotoxin, B subunit from <i>E. Coli</i> (LTB) under isofluorane anaesthetic

Once high antibody titres had been established by immunofluorescence (Section 3.5.4), two mice from each group were sacrificed by cardiac puncture. 1ml of PBS was washed through the nasal mucosa to collect any secretory immunoglobulins.

The rest of the mice were challenged with an intranasal inoculation of 50µl of HMPV174. They were weighed daily for signs of illness and on the 5<sup>th</sup> day sacrificed by cervical dislocation.

## 3.19 Immunohistochemistry

Immunohistochemistry was carried out using the Vector mouse on mouse (M.O.M.) immunodetection kit (Vector Laboratories). It is designed specifically to localise mouse primary monoclonal and polyclonal antibodies on mouse tissues avoiding the problem of anti – mouse secondary antibodies having to distinguish between the mouse primary antibody and endogenous mouse immunoglobulins in the tissue. The kit utilises a novel blocking agent and proprietary detection methodology to

significantly reduce the high background staining as a consequence of the endogenous mouse immunoglobulins.

### **3.19.1 Preparation of sections**

Tissues were fixed in 10% neutral buffered formalin before being paraffin embedded by the histopathology department at the Royal Victoria Infirmary (RVI). Sections were then cut to 3µm thick using a standard microtome. Cut sections were mounted onto Superfrost coated slides. These were then dried at 60°C for an hour followed by overnight at 37°C.

### **3.19.2 Histochemistry**

Sections were deparaffinised in xylene for 5 minutes and rehydrated in absolute alcohol followed by 95% alcohol before being washed in water. Slides were immersed in Haematoxylin Gill II (Surgipath) for 1 – 2 minutes and washed under water until the sections turned blue. The slides were then soaked in Eosin Y (Alcoholic solution, Surgipath) for 30 seconds to 1 minute before being washed under running water until the water ran clear. Sections were dehydrated through graded alcohols and cleared in xylene with subsequent mounting under coverslips in DPX.

### **3.19.3 Immunohistochemistry**

#### **Reagents**

Vector antigen retrieval solution (10mM Sodium citrate buffer – 20x)

42g Citric acid

21g Sodium hydroxide

Dissolve in 1 litre of distilled water and pH to 6.0

To use, dilute 75ml into 1425ml of distilled water and check pH.

Tris buffered saline (TBS) – 20x

121.14g Tris

160g Sodium chloride

Dissolve in 1 litre of distilled water and pH to 7.6.

To use, dilute 250ml into 4750ml of distilled water and check pH.

Sections were deparaffinised in xylene for 5 minutes and rehydrated in absolute alcohol followed by 95% alcohol before being washed in water. Antigen retrieval solution was brought to the boil in a pressure cooker using a hot plate. The slides were added and cooked at pressure (15lbs psi) for 1 minute before allowing them to cool for 5 to 10 minutes in water.

To block endogenous peroxidase, the sections were immersed in 3ml of 30% H<sub>2</sub>O<sub>2</sub> in 180ml of methanol at room temperature for 10 minutes and subsequently washed thoroughly in water followed by TBS for 10 minutes.

A streptavidin / Biotin blocking step was performed using the Streptavidin / Biotin blocking kit (Vector) following the manufacturers instructions. Sections were incubated for 1 hour in working solution of M.O.M. Ig blocking reagent prepared as described in the M.O.M. kit (Vector Laboratories). Sections were then soaked in TBS three times for 5 minutes before being incubated for 5 minutes with working solution of M.O.M. diluent prepared as described in the kit. Excess diluent was removed from the sections and the primary antibody (HMPV MAb pool), diluted at 1/50 in the diluent, was added for 30 minutes. Slides were washed as above before being incubated with working solution of M.O.M. biotinylated anti – mouse IgG reagent prepared as described in the kit for 30 minutes. A washing step followed as described previously after which, sections were incubated with Vectastain ABC reagent (Vector Laboratories) for 5 minutes. A final wash in TBS was performed before being developed in chromogen, using the Vector ImmPact DAB system (Vector Laboratories), where 1 drop of DAB substrate was added to 1ml of buffer. The sections were incubated for 5 to 10 minutes then washed in running water, counterstained with haematoxylin for 1 minute and ‘blue’ in Scotts Tap Water. Sections were dehydrated through graded alcohols and cleared in xylene with subsequent mounting under coverslips in DPX.

## **3.20 SDS Polyacrylamide gel electrophoresis (SDS PAGE)**

### **3.20.1 Preparation of reagents**

#### **3.20.1.1 8x stacking gel buffer**

6.05g Tris base

Dissolved in 50ml of distilled water and adjusted to pH 6.8

#### **3.20.1.2 6x sample buffer**

15g Glycerol

3mg Bromophenol blue

2.4g Sodium dodecyl sulphate (SDS)

7.5ml 8x stacking buffer

Volume was adjusted to 20ml with distilled water and stored in aliquots at -20°C.

Immediately before use; 50µl / ml of β-mercaptoethanol was added.

#### **3.20.1.3 2x Resolving gel buffer**

45.4g Tris base

1g SDS

Dissolved in 500ml of distilled water and adjusted to pH 8.8

#### **3.20.1.4 SDS running buffer**

28.8g Glycine

6.05g Tris base

2g SDS

Dissolved in 2 litres of distilled water

#### **3.20.1.5 10% Ammonium persulphate (APS) solution**

1g APS

9ml Distilled water

Stored at 4°C for 7 days

#### **3.20.1.6 Low concentration acrylamide solution**

25ml 40% Acrylamide 29: Bisacrylamide 1 solution (BDH)

75ml Distilled water

Stored at 4°C in foil wrapper

### **3.20.1.7 High concentration acrylamide solution**

40% Acrylamide 29: Bisacrylamide solution (BDH)

Stored at 4°C in foil wrapper

### **3.20.1.8 3% acrylamide solution**

9ml Low concentration acrylamide solution

15ml 2x resolving gel buffer

6ml Distilled water

The solution was gently mixed and degassed using a vacuum pump for 5 minutes. Immediately before pouring, 240µl of 10% APS solution and 15µl N,N,N,N-tetramethylethylenediamine (TEMED) were added and the solution gently mixed.

### **3.20.1.9 18% acrylamide solution**

3.9g Sucrose

11.7ml High concentration acrylamide solution

13ml 2x resolving gel buffer

The solution was gently mixed and degassed using a vacuum pump for 5 minutes. Immediately before pouring, 180µl of 10% APS solution and 10µl TEMED were added and the solution gently mixed.

### **3.20.2 Pouring gradient gels**

Gels were poured into Protean II gel moulds (Bio-Rad Laboratories) using a Jencons-PLS gradient mixer. A magnetic flea was placed into the column of the gradient mixer containing the 18% solution and as the acrylamide flowed into the mould, the concentration of acrylamide gradually decreased from 18% to 3% at the top of the gel. Once poured, a 15-well comb was gently inserted into the top of the gel and the gel was allowed to set. The wells were subsequently washed five times with distilled water and once with 1 x stacking gel buffer.

### **3.20.3 Running gradient gels**

Samples were prepared by adding 5µl of protease inhibitor to frozen stocks which were then rapidly thawed. Samples were mixed 6:1 in 6x sample buffer and heated to 100°C for 3 minutes. Gels were removed from pouring apparatus and wells washed gently with SDS running buffer before being transferred to a Protean II tank (Bio-Rad Laboratories) which was half filled with SDS running buffer. 60µl of each sample was loaded into the wells of the gel along with 10µl of molecular weight markers (Fermentas) and 60µl of 1x sample buffer to all unused wells. The tank was placed on a magnetic stirrer connected to a Grant cooling system set to 0.5°C. Using an electrophoresis power supply (EPS) 600, a current of 27mA, 600 V, 100 W was applied to the gel overnight.

## **3.21 Western blot of SDS PAGE gels**

### **3.21.1 Preparation of reagents**

#### **3.21.1.1 Tris-glycine protein transfer buffer**

3.03g Tris base

14.4g Glycine

200ml Methanol

Dissolved in 650ml of distilled water and finally adjusted to 1 litre. Stored at 4°C.

#### **3.21.1.2 10x phosphate buffered saline**

160g Sodium Chloride

4g Potassium dihydrogen orthophosphate

23g Di-sodium hydrogen orthophosphate

4g Potassium chloride

Dissolved in 2 litres of distilled water and adjusted to pH 6.8.

#### **3.21.1.3 Blocking buffer 1**

200ml PBS containing 0.1% Tween-20 (PBS/T)

10g Marvel milk powder

2g Bovine serum albumin (BSA) (BDH)

The solution was filtered through Whatman glass microfibre filter paper before use.

#### **3.21.1.4 Blocking buffer 3**

360ml PBS/T

40ml Heat inactivated foetal calf serum

4g BSA

The solution was filtered through a 0.2 $\mu$ M vacuum filter and stored at -20°C.

#### **3.21.2 Transfer of SDS PAGE gels**

The gel was washed 3x 20 minutes in tris-glycine protein transfer buffer whilst Hybond-P Polyvinylidene fluoride (PVDF) transfer membrane (GE healthcare) was cut to the exact size of the SDS gel and soaked in methanol for 5 minutes followed by 20 minutes in protein transfer buffer. The gel and membrane were sandwiched together in a Bio-Rad transplant apparatus (Bio-Rad Laboratories) with 4 sheets of 3mm Whatman filter paper and one sponge either side of them. The transfer apparatus filled with protein transfer buffer was attached to the Grant cooling system set at 0.5°C. 120 V was applied to the equipment using a BIO-Rad 200 power pack for 5 hours. After transferring, the PDVF membrane was washed 3 times in distilled water and blocked overnight in blocking buffer 1 at room temperature.

### **3.22 Staining western blots with Enhanced Chemiluminescence (ECL) kit**

#### **3.22.1 Preparation of reagents**

##### **3.22.1.1 1x Developer solution**

390ml Distilled water

110ml GBx concentrated developer (Kodak, from Sigma)

Kept in foil wrapper and used within 4 days

##### **3.22.1.2 1x Fixer solution**

390ml Distilled water

110ml GBx concentrated fixer (Kodak, from Sigma)

Kept in foil wrapper and used within 4 days

### **3.23 ECL plus western blotting detection system**

#### **ECL detection solution**

20ml ECL solution A (Amersham Biosciences)

500µl ECL solution B (Amersham Biosciences)

Solutions were mixed just before use and stored wrapped in tin foil until required.

The PVDF membrane was washed 2 x 5 minutes in PBS/T and subsequently incubated in 50ml of primary antibody (mouse monoclonal or mouse monospecific antibody) diluted in blocking buffer 3 for 90 minutes at room temperature on an orbital shaker. The membrane was washed 3 x 15 minutes and 1 x 30 minutes in PBS/T, followed by a 5 minute wash in blocking buffer 3. Dilutions of secondary antibody were made in blocking buffer 3 (1/6000 RAMP) and incubated on the membranes for 90 minutes at room temperature on an orbital shaker. The membrane was rinsed 3 x 15 minutes in PBS/T, blotted dry on filter paper and incubated in ECL detection solution for 5 minutes. The membrane was blotted again before being wrapped in clingfilm and transferred to the dark room. The membrane was then exposed to photographic film (Hyperfilm ECL, Amersham Biosciences) for between 10 seconds and 1 minute, depending on the strength of the luminescence. The films were developed for 2 minutes in Gbx developer followed by 2 minutes in Gbx fixer and were finally rinsed in distilled water. Films were left to dry overnight at room temperature.

## Chapter 4: Production of recombinant vaccinia virus expressing HMPV glycoproteins

### 4.1 Preparation of polyclonal mouse anti-sera to HMPV174, HMPV145 and HMPV128

Three groups of 3 mice were immunised fortnightly with an initial intranasal inoculation (Section 3.10.1) followed by two subcutaneous inoculations (Section 3.10.2) with either HMPV174, 145 or 128 grown in 16HBE140 at a titre of  $5 \times 10^5$  ffu/ml. Thirty-five days after the initial intranasal inoculation, the serum antibody titres (Table 4.1) were established by titrating the tail bleeds by indirect immunofluorescence, staining on HMPV infected cells in comparison with uninfected cells. Mice immunised with HMPV128 received an additional subcutaneous inoculation on day 42 to boost serum antibody titres.

**Table 4.1 Antibody titres of tail bleeds and cardiac punctures from HMPV immunised mice against the homologous strain.**

Inoculum	Titre ( $\log_{10}$ )		
	Tail bleed 1	Tail bleed 2	Final bleed
	Day 35	Day 49	
HMPV174	2.81	-	2.81
HMPV145	2.81	-	2.81
HMPV128	2.2	2.51	2.51

All serum collected was absorbed with 16HBE140 cells (Section 3.5.3) to remove any cross reactive anti-human antibodies before being used for the detection of HMPV glycoprotein expression.

### 4.2 Immunofluorescence

#### 4.2.1 Polyclonal anti-serum

All sera collected were tested for cross reactivity against strains HMPV174 and HMPV145 (both clustered with sub-group A2 (Ingram, 2006)) and HMPV128

(clustered with sub-group B1 (Ingram, 2006)) and the results are presented in Table 4.2.

**Table 4.2 Cross reactivity of polyclonal anti-HMPV mouse serum on HMPV infected 16HBE140 cell cultures.**

Virus	Mouse polyclonal anti-HMPV antibody (titre log <sub>10</sub> )		
	Anti-174	Anti-145	Anti-128
<b>HMPV174</b>	2.81	2.51	2.2
<b>HMPV145</b>	2.51	2.81	2.2
<b>HMPV128</b>	2.51	2.51	2.51

Whilst all polyclonal serum cross reacted against all tested strains of HMPV, anti HMPV128 polyclonal serum had generally lower titres in comparison to HMPV174 and 145.

### **4.3 Production of recombinant pTM1 HMPV clones**

In order to produce recombinant vaccinia virus clones expressing the different glycoproteins of HMPV174, 145 and 128, the glycoprotein genes were first amplified by reverse transcription PCR from the viral RNA. The PCR products were inserted into the vector, pTM1, to allow recombination to occur between the thymidine kinase regions in the plasmid and the vaccinia virus genome. Once cloned into pTM1, all genes were sequenced using the forward and reverse primers used for cloning and cross referenced with sequences on the NCBI database for anomalies.

The vaccinia virus recombinants were finally coinfecting with VTF7.3 to allow authentic expression of the gene.

#### **4.3.1 Plasmid pTM1**

Initially, pTM1 DNA was transformed into competent *E. coli*, TG1, as described in section 3.7.7. Plasmid bearing colonies were identified using a miniprep kit as described in section 3.7.8. The eluted DNA was digested with restriction endonucleases *NcoI* and *XhoI* (Section 3.7.5), before being visualised by ethidium bromide staining following gel electrophoresis (Section 3.6.6). A positive colony was

inoculated into 500ml of LB + ampicillin broth and pTM1 DNA was prepared using a QIAGEN maxiprep kit as described in section 3.7.9. The concentration of plasmid DNA was determined as 3.2 mg/ml. A restriction digest was performed on the plasmid using enzymes *NcoI* and *XhoI* and the results are presented in Figure 4.1. An expected band of 5357bp corresponding to the digested pTM1 plasmid was present (lane 3).



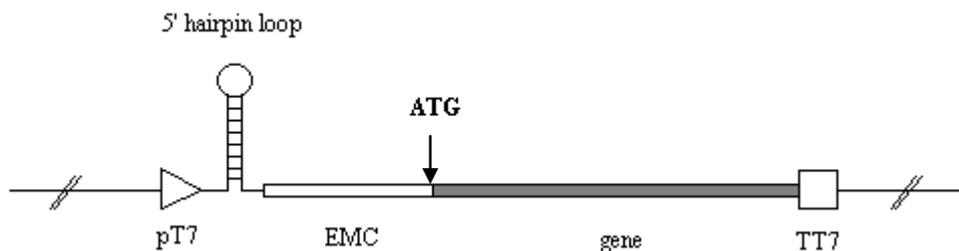
**Figure 4.1 Agarose gel electrophoresis of pTM1 restriction digests.**

The lanes were loaded as follows; 1 - 1kb molecular marker, 2 - 1 $\mu$ g pTM1 undigested, 3 - 1 $\mu$ g pTM1 digested with *NcoI* and *XhoI*.

### 4.3.2 HMPV128 F and G glycoproteins

#### 4.3.2.1 PCR primer design

In the VTF7.3 system, mRNA expressed from the T7 promoter requires a hairpin loop to stabilise the transcript. Since the hairpin loop prevents capping of the transcript and potentially ribosome binding and scanning, an encephalomyocarditis virus independent ribosomal entry site (EMC) sequence is also required for efficient translation of non-capped transcripts (see Figure 4.2) (Elroy-Stein *et al.*, 1989; Fuerst and Moss, 1989). The plasmid, pTM1 (Figure 1.4), possesses an AUG start codon in the *NcoI* restriction enzyme site downstream from the T7 promoter and EMC site. Utilising this start codon to represent the AUG start codon of the target gene would allow efficient translation of the gene when inserted into the vaccinia virus genome.



**Figure 4.2 Structure of the expression cassette in pTM1**

Therefore, to insert the glycoprotein genes of HMPV into pTM1, the primer sequences must incorporate the restriction endonuclease site *NcoI* at the 5' end over the start codon and another restriction enzyme site, present in the MCS of pTM1, at the 3' end.

The *NcoI* site contains the start codon, bases 3 to 5, with the sixth base being a guanosine. Therefore, each gene inserted into the plasmid must possess a guanosine after the start codon to be recognised by the restriction enzyme. However, as this is not the case with both the F and SH genes of HMPV, other restriction endonucleases could be substituted that would reproduce the same overhang as *NcoI* (Table 4.3), allowing ligation between the 5' end of the gene and the *NcoI* digested plasmid.

**Table 4.3 Restriction enzyme sites and overhangs**

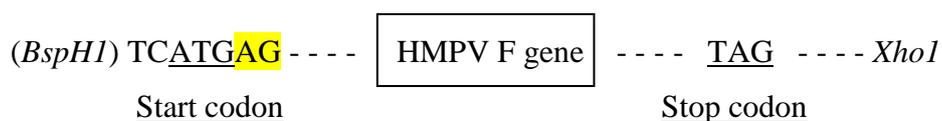
Restriction enzyme	Sequence (5' to 3')	Overhang	HMPV gene
<i>NcoI</i>	C / C A T G G G G T A C / C	CATG	G
<i>BspHI</i>	T / C A T G A A G T A C / T	CATG	SH
<i>PciI</i>	A / C A T G T T G T A C / A	CATG	F

#### 4.3.2.1.1 PCR of HMPV128 F gene

Searching the NCBI nucleotide database for HMPV F gene sequences from the B subtype, yielded several sequences that all shared a common 3' end. The start codon is followed by TC and so the restriction endonuclease *PciI* could be used for insertion into pTM1. For PCR design, the first 29 bases from the AUG start codon were used as a sequence for the forward primer. The 5' end also shared close homology between the different database sequences and 24 bases including the TAG stop codon were used as a complimentary sequence for the reverse PCR primer. The HMPV F gene was searched for restriction endonucleases *PciI* and *XhoI* (a restriction enzyme site present in the MCS of pTM1) however, the *PciI* enzyme cuts within the gene. Since no other restriction enzymes were available, during primer design the first 2 nucleotides (thymine and cytosine) in the sequence after the start codon were

substituted for adenine and guanine. This alteration maintains the integrity of the amino acid sequence, conserving the serine residue (UCU and AGU encode serine), but allows the restriction enzyme *BspHI* to be used for digestion of the gene at the 5' end.

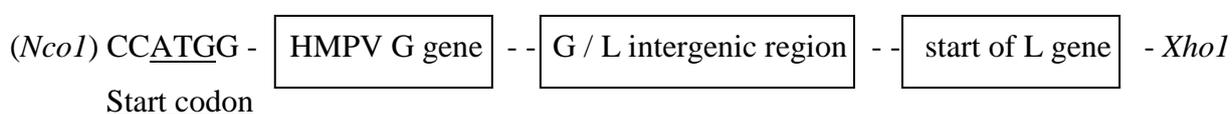
Therefore, the PCR product will incorporate the *BspHI* upstream of the HMPV F gene and the *XhoI* downstream to allow correct orientation of the gene into the pTM1 plasmid.



The reverse transcription reaction was carried out using the reverse primer as this will transcribe mRNA, which is more abundant than genome in infected cell lysate. RT-PCR was performed with primer set 1 (Table 3.6) using the conditions described in Table 3.9 and 3.12 (RT step and AliT2).

#### 4.3.2.1.2 PCR of HMPV128 G gene

A search on the NCBI nucleotide database yielded several sequences for the HMPV G gene, where the B subtype strains showed high homology within the first 90 nucleotides. The AUG is followed by a G allowing *NcoI* to be used for cloning into pTM1. The forward primer included the first 25 nucleotides including the AUG start codon and an *NcoI* site over the AUG start codon. For the reverse primer, there was not enough homology between the sequences in the database to obtain a consensus sequence so the primer was designed around the start of the adjacent L gene. The first 27 nucleotides including the start codon were used as a primer template. The entire gene was checked for the presence of restriction endonuclease sites, and *NcoI* and *XhoI* were not found allowing these sites to be added to the forward and reverse primers respectively.

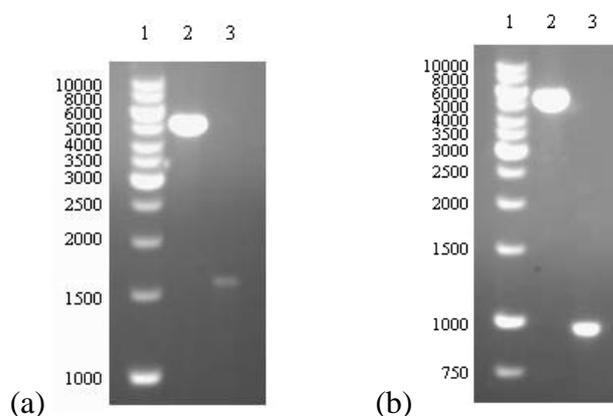


As the reverse primer is located in a separate gene, the forward primer was used in the reverse transcription of virus genome, as the transgenic region is required for amplification. RT-PCR was performed with primer set 2 (Table 3.6) and was carried out according to the RT step and AliT2 program (Table 3.9 and 3.12).

#### 4.3.2.2 Digestion of HMPV128 F and G gene PCR products and pTM1 plasmid

RT-PCR produced a band of approximately 1600bp for the F gene reaction (expected 1620 bp) and 900bp for the G gene reaction (expected 901bp) when the reaction mix was run on a 1% agarose gel. The products were cut from the gel and purified as described in section 3.6.7.

To facilitate ligation of the genes into pTM1, gel purified HMPV F gene product (Section 3.6.7) was digested with restriction endonucleases *BspHI* and *XhoI*, whilst HMPV G gene product and pTM1 were digested with *NcoI* and *XhoI*. Reactions were incubated for 3 hours and subsequently run on a 1% agarose gel to be observed with ethidium bromide staining and ultraviolet light. The results are shown in Figure 4.3.



**Figure 4.3 Plasmid pTM1, (a) F gene and (b) G gene restriction digest.**

The lanes were loaded as follows; 1 - 1kb molecular marker, 2 - pTM1 digested with *NcoI* and *XhoI*, 3(a) F gene digested with *BspHI* and *XhoI* 3(b) G gene digested with *NcoI* and *XhoI*.

Bands of appropriate size for the F gene (1620 bp, Figure 4.3a) and the G gene (901 bp, Figure 4.3b) and a band of approximately 5357 base pairs (the pTM1 plasmid) were identified and removed with a clean scalpel. Using a QIAquick gel extraction kit, bands were purified and eluted in EB buffer (Section 3.6.7), ready for ligation.

### 4.3.2.3 Ligation and transformation of pTM1 with HMPV128 gene products

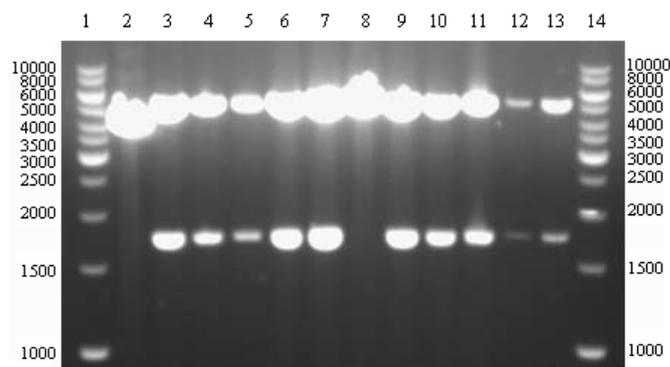
To ensure a successful transformation, 18 different ligations were carried out as described in section 3.7.6. Once ligation products were produced, they were transformed into *E. coli* TG1 (Section 3.7.7) and spread onto LB agar plates containing ampicillin. The results are shown in Table 4.4.

**Table 4.4** Number of colonies counted after transformation of bacteria with pTM1.128F and pTM1.128G.

Reaction	pTM1.128F	pTM1.128G
Vector only 2µl	2	0
Vector only 8µl	2	0
Vector only 20µl	2	1
Vector + insert 1:1, 2µl	7	10
Vector + insert 1:1, 8µl	20	25
Vector + insert 1:1, 20µl	13	54
Vector + insert 2:5, 2µl	17	0
Vector + insert 2:5, 8µl	39	0
Vector + insert 2:5, 20µl	75	0
Vector + insert 1:3, 2µl	8	8
Vector + insert 1:3, 8µl	15	24
Vector + insert 1:3, 20µl	32	26
Vector + insert 1:5, 2µl	25	10
Vector + insert 1:5, 8µl	70	38
Vector + insert 1:5, 20µl	83	51
No DNA control	0	0
No DNA control	0	0
No DNA control	0	0

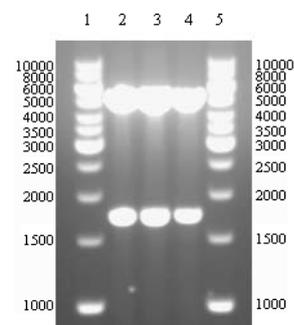
One colony from each successful plate was taken and grown up overnight in 5ml of LB medium containing ampicillin. To check the validity of the clones, plasmid DNA was prepared using a QIAgen miniprep spin column as described in section 3.7.8. The

eluted DNA was digested according to Table 3.13. The product was resolved on a 1% agarose gel and the results for pTM1.128F are presented in Figure 4.4.



**Figure 4.4 Restriction digest of transformation of pTM1 with the HMPV128 F gene.**

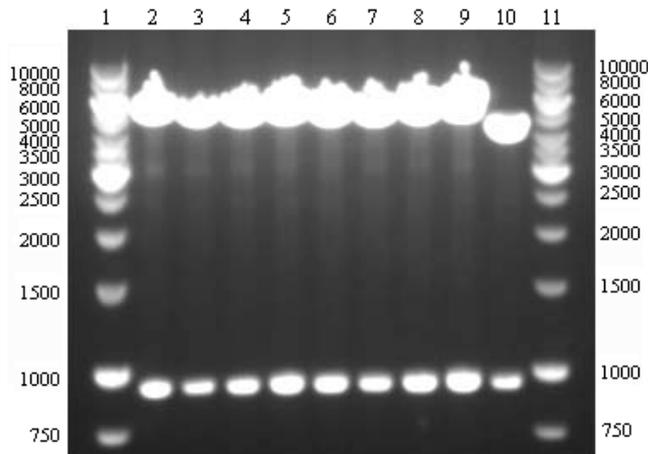
The lanes are as follows; 1 - 1Kb molecular marker, 2 to 13 – various colonies picked, 14 – 1Kb molecular marker.



**Figure 4.5 Restriction digest of pTM1.128F clone 5.**

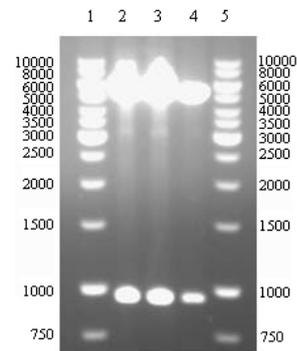
The lanes are as follows; 1 - 1Kb molecular marker, 2 to 4 - colonies picked from one clone, 5 – 1Kb molecular marker.

All but two of the twelve transformed bacterial clones contained two bands, one just over 5kbp, corresponding to the pTM1 plasmid and another approximately 1.7kbp corresponding to 1620bp for the F gene and approximately 100bp lying between the end of the F insert and the *KpnI* site in the vector (Figure 1.4). Based on the gel in Figure 4.4, one clone (pTM1.128F clone 5) was picked and recloned onto LB agar with ampicillin. Three colonies were inoculated into LB medium, containing ampicillin, overnight. DNA was prepared as above and digested with both *KpnI* and *XhoI* restriction enzymes to ensure purity of the culture (Figure 4.5). One of these clones (pTM1.128F clone 5.2) was chosen for large scale preparation of a plasmid DNA maxiprep (see Section 3.7.9). The concentration of DNA eluted was 2.5 mg/ml.



**Figure 4.6 Restriction digest of transformation of pTM1 with the HMPV128 G gene.**

The lanes are as follows; 1 - 1Kb molecular marker, 2 to 10 – various colonies picked, 11 – 1Kb molecular marker.

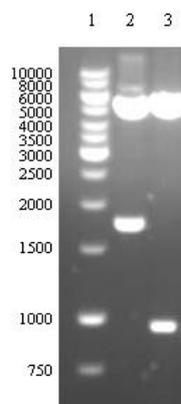


**Figure 4.7 Restriction digest of pTM1.128G clone 8.**

The lanes are as follows; 1 - 1Kb molecular marker, 2 to 4 - colonies picked from one clone, 5 – 1Kb molecular marker.

The results for pTM1.128G are presented in Figure 4.6. All nine transformed bacterial clones contained two bands, one just over 5kbp corresponding to the pTM1 plasmid and another at approximately 900bp corresponding to the G gene. One clone (pTM1.128G clone 8) was picked and recloned similarly to the F gene above, and the DNA digested with restriction enzymes *NcoI* and *XhoI* (Figure 4.7). Using a QIAGEN maxiprep kit, large scale plasmid DNA prep was prepared as above with pTM1.128G clone 8.2 and produced a yield of 3.1 mg/ml total DNA.

To ensure the plasmid DNA was eluted correctly, both pTM1.128F clone 5.2 and pTM1.128G clone 8.2 DNA preparations were double digested with restriction enzymes *KpnI* and *XhoI*, and *NcoI* and *XhoI* respectively. The results are presented in Figure 4.8.



**Figure 4.8 Restriction digest of pTM1.128F clone 5.2 and pTM1.128G clone 8.2 maxiprep.**

Lanes are as follows; 1 - 1Kb molecular marker, 2 - pTM1.128F5.2, 3 - pTM1.128G8.2

### 4.3.3 HMPV145 F and G glycoprotein

#### 4.3.3.1 Primers

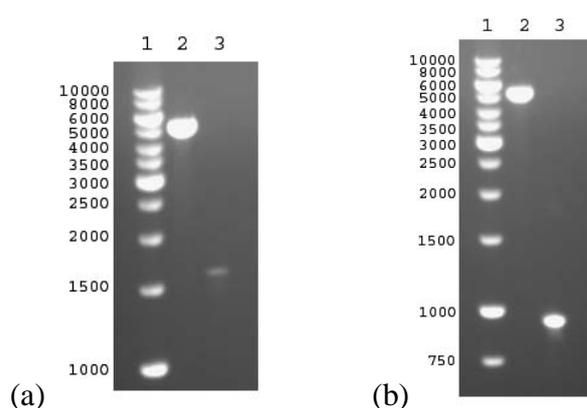
HMPV145 clusters with subtype A2 strains in phylogenetic analyses (Ingram, 2006). As the sequence homology between the A and B subgroups of HMPV is high, the subtype B HMPV128 F primers (primer set 1, Table 3.6) were used to amplify the subtype A HMPV strain 145 F gene using the RT step and AliT2 conditions (Table 3.9 and 3.12).

#### 4.3.3.2 PCR of HMPV145 G gene

For the G gene of HMPV145, HMPV174 G primers (primer set 2, Table 3.8) were modified from Robinson, 2007 where *NcoI* and *XhoI* restriction enzyme sites were added to the forward and reverse primers respectively (as described in Section 4.3.2.1.2). The subtype A HMPV strain 145 G gene was amplified with primer set 1 (Table 3.7) using the RT step and AliT2 conditions (Table 3.9 and 3.12).

#### 4.3.3.3 Digestion of HMPV145 F and G gene PCR products and pTM1 plasmid

HMPV145 F and G gene products were purified as described in section 3.6.7 and were digested with restriction enzymes specified in Table 3.13. Reactions were incubated for 3 hours and the products run on a 1% agarose gel. The results are shown in Figure 4.9.



**Figure 4.9. Plasmid pTM1, (a) HMPV145 F gene and (b) HMPV145 G gene restriction digest.**

The lanes were loaded as follows; 1 - 1kb molecular marker, 2 - pTM1 digested with *NcoI* and *XhoI*, 3 - F gene digested with *BspHI* and *XhoI* / G gene digested with *NcoI* and *XhoI*.

A band corresponding to the 1620 base pairs for the F gene (a) and the 901 base pairs for the G gene and a band of approximately 5357 base pairs (the pTM1 plasmid) were

identified and removed with a clean scalpel. Using a QIAquick gel extraction kit, bands were purified and eluted in EB buffer (section 3.6.7), ready for ligation.

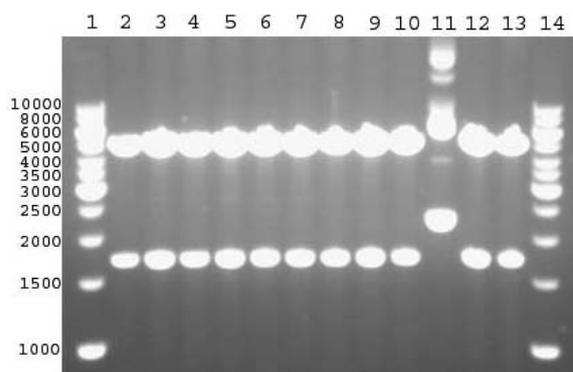
#### 4.3.3.4 Ligation and transformation of pTM1 with HMPV145 gene products

18 different ligation reactions were carried out as described in section 3.7.6. Once ligation products were produced, they were transformed into *E. coli* TG1 (Section 3.7.7) and spread onto LB agar plates containing ampicillin. The results are shown in Table 4.5.

**Table 4.5** Number of colonies counted after transformation of bacteria with pTM1.145F and pTM1.145G.

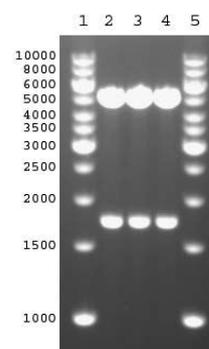
Reaction	pTM1.145F	pTM1.145G
Vector only 2µl	0	0
Vector only 8µl	0	0
Vector only 20µl	0	0
Vector + insert 1:1, 2µl	9	69
Vector + insert 1:1, 8µl	33	262
Vector + insert 1:1, 20µl	45	too many to count
Vector + insert 2:5, 2µl	82	272
Vector + insert 2:5, 8µl	167	568
Vector + insert 2:5, 20µl	194	too many to count
Vector + insert 1:3, 2µl	15	82
Vector + insert 1:3, 8µl	57	235
Vector + insert 1:3, 20µl	122	too many to count
Vector + insert 1:5, 2µl	55	148
Vector + insert 1:5, 8µl	223	272
Vector + insert 1:5, 20µl	248	456
No DNA control	0	0
No DNA control	0	0
No DNA control	0	0

One colony from each successful plate was taken and grown up as described previously. Plasmid DNA obtained from a QIAgen miniprep was digested according to Table 3.13. The results for pTM1.145F are presented in Figure 4.10.



**Figure 4.10 Restriction digest of transformation of pTM1 with the HMPV145 F gene.**

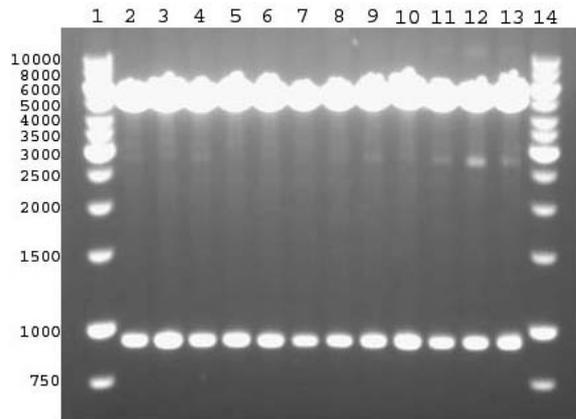
The lanes are as follows; 1 - 1Kb molecular marker, 2 to 13 – various colonies picked, 14 – 1Kb molecular marker.



**Figure 4.11 Restriction digest of pTM1.145F clone 8.**

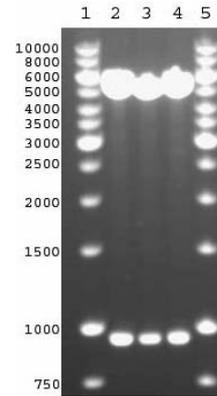
The lanes are as follows; 1 - 1Kb molecular marker, 2 to 4 - colonies picked from one clone, 5 – 1Kb molecular marker.

All but one of the twelve transformed bacterial clones contained two bands, one just over 5kbp representing the pTM1 plasmid and the other, approximately 1.7kbp, corresponding to the F gene (Figure 4.10). One of these eleven clones (pTM1.145F clone 8) was picked and recloned on LB agar ampicillin plates followed by growth in LB medium containing ampicillin overnight. DNA was prepared as before and digested with both *KpnI* and *XhoI* restriction endonucleases to ensure stability of the clone (Figure 4.11). One clone (pTM1.145F clone 8.1) was subsequently used for maxiprep of recombinant plasmid DNA (see Section 3.7.9). The concentration of DNA yielded 382.5µg/ml.



**Figure 4.12 Restriction digest of transformation of pTM1 with the HMPV145 G gene.**

The lanes are as follows; 1 - 1Kb molecular marker, 2 to 13 – various colonies picked, 14 – 1Kb molecular marker.

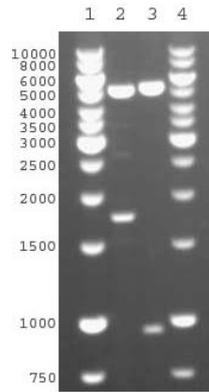


**Figure 4.13 Restriction digest of pTM1.145G clone 2.**

The lanes are as follows; 1 - 1Kb molecular marker, 2 to 4 - colonies picked from one clone, 5 – 1Kb molecular marker.

The results for pTM1.145G are presented in Figure 4.12. All twelve transformed bacterial clones contained two bands, one just over 5kbp representing the pTM1 plasmid and the other just over 900bp, corresponding to the G gene. Clones 11 to 13 contained an additional faint band at approximately 3300bp, however it is not clear what they represent. One clone (pTM1.145G clone 2) was picked and recloned similarly to the F gene above, and the DNA digested with restriction enzymes *NcoI* and *XhoI* (Figure 4.13). Preparation of plasmid DNA of pTM1.145G clone 2.3 was carried out using a QIAGEN maxiprep kit (Section 3.7.9) and produced a yield of 425µg/ml total DNA.

To ensure the plasmid DNA was eluted correctly, both pTM1.145F clone 8.1 and pTM1.145G clone 2.3 DNA preparations were double digested with restriction enzymes *KpnI* and *XhoI*, and *NcoI* and *XhoI* respectively. The results are presented in Figure 4.14.

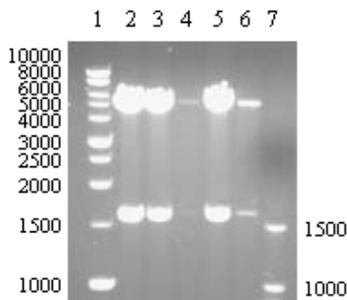


**Figure 4.14 Restriction digest of pTM1.145F clone 8.1 and pTM1.145G clone 2.3 maxiprep.**

Lanes are as follows; 1 - 1Kb molecular marker, 2 - pTM1.145F8.1, 3 - pTM1.145G2.3

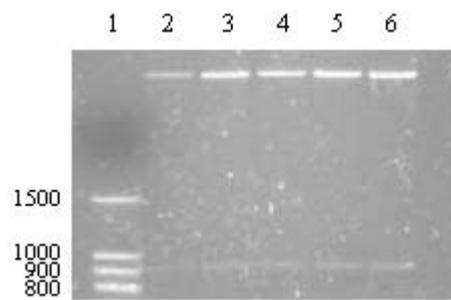
#### 4.3.4 HMPV174 F and G glycoproteins

*E. coli*, TG1, transformed with pTM1 plasmids containing the F and G gene of HMPV174, were kindly provided by Dr Mark Robinson. The genes were integrated into the MCS of the pTM1 plasmid using restriction endonucleases *Bam*HI and *Eco*RI (see Figure 1.4). Both preparations were recloned as before and DNA was once again extracted from the cultures and digested using restriction enzymes *Bam*HI and *Eco*RI. The results are presented in Figure 4.15 and Figure 4.16.



**Figure 4.15. Restriction digest of pTM1.174F clone 6.**

The lanes are as follows; 1 - 1Kb molecular marker, 2 to 6 - colonies picked from one clone, 7 - 100bp molecular marker.

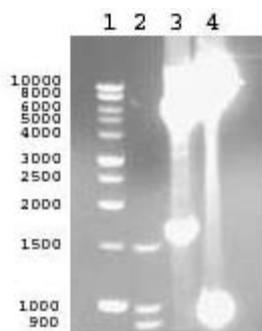


**Figure 4.16. Restriction digest of pTM1.174G clone 2.**

The lanes are as follows; 1 - 100bp molecular marker, 2 to 6 - colonies picked from one clone.

All transformed bacterial colonies were positive for both pTM1 (5357bp) and the gene insert (F gene at 1620bp and G gene at 901bp). Maxipreps of the recombinant plasmids were prepared (Section 3.7.9) and produced a yield of 640µg/ml total DNA for pTM1.174F clone 6.1 and 1.506mg/ml total DNA for pTM1.174G clone 3.6.

These plasmids were digested with restriction endonucleases *Bam*HI and *Eco*RI for 3 hours before being visualised by ethidium bromide staining following agarose gel electrophoresis (Figure 4.17).



**Figure 4.17 Restriction digest of pTM1.174F clone 6.1 and pTM1.174G clone 3.6 maxiprep.**

Lanes are as follows; 1 - 1Kb molecular marker, 2 – 100bp molecular marker, 3 - pTM1.174F clone 6.1, 4 - pTM1.174G clone 3.6.

#### 4.4 Sequencing

Once cloned into pTM1, all genes were sequenced. As a comparison, each gene was evaluated for both its nucleotide and amino acid identity with the corresponding genes from either HMPV128, HMPV145 or HMPV174 to remove the possibility of cross contamination (Table 4.6).

These genes were also evaluated for both their nucleotide and amino acid identity with strains from the NCBI database representing all four lineages (Table 4.7). These were an A1 strain JPS03/180 (Accession number AY530092), an A2 strain CAN97/83 (Accession number AY297749), a B1 strain JPS02/76 (Accession number AY530089) and a B2 strain CAN98/75 (Accession number AY297748).

**Table 4.6 Percentage of amino acid or nucleotide sequence identity between HMPV128, HMPV145 and HMPV174 F and G genes**

HMPV genes	Percent amino acid sequence identity (percent nucleotide sequence identity <sup>†</sup> )		
	HMPV128 vs HMPV145	HMPV128 vs HMPV174	HMPV145 vs HMPV174
<b>F</b>	98 (97)	93 (83)	93 (82)
<b>G</b>	68 (92)	31 (59)	34 (59)

<sup>†</sup> See footnote Table 4.7

**Table 4.7 Percentage of amino acid or nucleotide sequence identity between HMPV128, HMPV145 and HMPV174 genes and other HMPV strains from the NCBI database**

<b>HMPV128</b> genes compared	<b>Percent amino acid sequence identity (percent nucleotide sequence identity<sup>†</sup>)</b>			
	<b>JPS03/180 (A1)</b>	<b>CAN97/83 (A2)</b>	<b>JPS02/76 (B1)</b>	<b>CAN98/75 (B2)</b>
<b>F<sup>a</sup></b>	94 (84)	94 (83)	99 (98)	98 (94)
<b>G<sup>b</sup></b>	30 (57)	34 (61)	92 (94)	65 (79)
<b>HMPV145</b>				
genes compared				
<b>F<sup>a</sup></b>	93 (84)	93 (83)	98 (97)	97 (93)
<b>G<sup>c</sup></b>	30 (61)	36 (61)	70 (94)	55 (79)
<b>HMPV174</b>				
genes compared				
<b>F<sup>a</sup></b>	98 (93)	98 (96)	93 (83)	94 (83)
<b>G<sup>d</sup></b>	64 (80)	84 (91)	32 (59)	35 (61)

<sup>†</sup> Amino acid sequence identities were calculated based on the complete predicted proteins; in the case of G, overhangs on the carboxy-terminal side of the alignments due to length differences were not included in the calculations. Nucleotide sequence identities are shown in parentheses and are based on the protein-coding sequence exclusive of non-coding sequences.

<sup>a</sup> The F genes from HMPV128, HMPV145 and HMPV174 were sequenced in both directions using primer set 1 (Table 3.6) (Appendix 1).

<sup>b</sup> HMPV128 G gene was sequenced using primer set 2 (Table 3.6) (Appendix 2)

<sup>c</sup> HMPV145 G gene was sequenced using primer set 1 (Table 3.7) (Appendix 2)

<sup>d</sup> HMPV174 G gene was sequenced using primer set 2 (Table 3.8) (Appendix 2)

Whilst HMPV128 appears to most closely resemble JPS02/76 B1 and HMPV174 most closely resembles CAN97/83 A2 in both genes, the F and G genes of HMPV145 appear to be most closely related to JPS02/76 B1 despite the previous report by Ingram (2006) that the N gene sequence of HMPV145 clustered with sub-group A2. Therefore, the N gene was sequenced again and evaluated for its nucleotide and amino acid identity with strains from the NCBI database (Table 4.8). Sequence analysis confirms the results by Ingram (2006) with HMPV145 N gene most closely resembling CAN97/83 A2.

**Table 4.8 Percentage of amino acid or nucleotide sequence identity between HMPV145 N gene and other HMPV strains from the NCBI database**

HMPV145 genes compared	Percent amino acid sequence identity (percent nucleotide sequence identity <sup>†</sup> )			
	JPS03/180 (A1)	CAN97/83 (A2)	JPS02/76 (B1)	CAN98/75 (B2)
N*	98 (94)	98 (97)	98 (88)	98 (89)

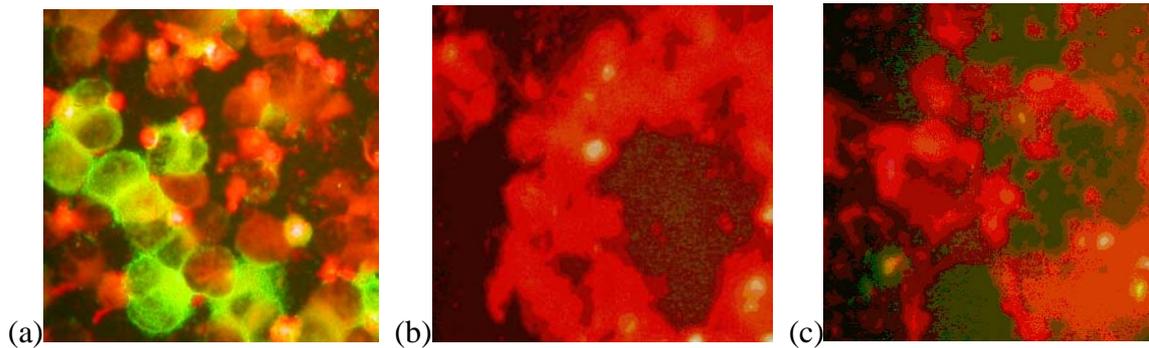
\* HMPV145 N gene was sequenced using primer set N-LN F / N-LN R (Table 3.10) (Appendix 4)

#### **4.5 Transient expression of HMPV.pTM1 clones in vaccinia virus**

Recombinant vaccinia viruses were generated from only HMPV174 (A2 strain) and HMPV128 (B1 strain), however, HMPV145 (A2 strain) was utilised in the transient expression system.

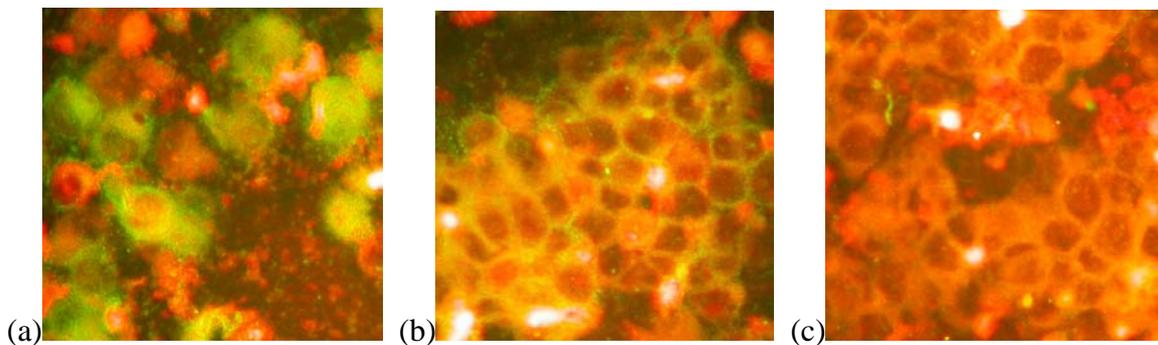
To ensure the constructs had the ability to allow authentic expression of the HMPV glycoproteins in the vaccinia virus system, DNA from pTM1.174F clone 6.1, pTM1.174G clone 3.6, pTM1.145F clone 8.1, pTM1.145G clone 2.3, pTM1.128F clone 5.2 and pTM1.128G clone 8.2 were transfected into VTF7.3 infected HeLa cells (section 3.5.12). After 21 hours, the cells were tested for expression of F and G antigen by immunofluorescence staining with an anti-F monoclonal antibody, MAb24, and homologous and heterologous mouse anti-HMPV polyclonal serum (see Section 3.2.1.2) diluted at 1/20 in PBS (Figures 4.18 and 4.19 and Table 4.9).

Specific binding of both MAb24 and the mouse polyclonal anti-HMPV serum to the transiently expressed HMPV F glycoprotein of all three F clones was observed, indicating the F glycoproteins from all strains of HMPV are expressed and are antigenically similar. The control VTF7.3 infected HeLa cells which were not transfected with plasmid DNA and uninfected, non-transfected HeLa cells showed no staining with MAb24 but there was slight non-specific binding of the mouse polyclonal anti-HMPV serum to both negative controls. Therefore, a staining score of  $\geq ++$  was taken as indicative of specific antibody binding with the polyclonal antibodies.



**Figure 4.18** Example of immunofluorescence staining in transiently expressed (a) pTM1.174F, (b) VTF7.3 infected HeLa cells (c) and HeLa cells all stained with MAb24.

Staining of the three G glycoprotein expressing clones with mouse polyclonal anti-HMPV antibodies was strain specific suggesting the G glycoprotein from all three clones was expressed but that the G glycoprotein is highly antigenically variable. No staining of the three G glycoprotein expressing clones occurred with MAb24, confirming that the G glycoprotein is expressed alone.



**Figure 4.19** Example of immunofluorescence staining in transiently expressed (a) pTM1.174G, (b) VTF7.3 infected HeLa cells (c) and HeLa cells all stained with mouse anti-HMPV174 polyclonal antibody.

**Table 4.9 Reactivity of mouse anti-HMPV polyclonal serum with transiently expressed HMPV F and G genes from HMPV174, 145 and 128.**

Virus	Mouse polyclonal anti-HMPV antibody			MAb24	
	Anti-174	Anti-145	Anti-128		
<b>pTM1.174</b>	<b>G clone 3.6</b>	++	+	+	-
	<b>F clone 6.1</b>	+++	+++	++	++
<b>pTM1.145</b>	<b>G clone 2.3</b>	-/+	++	+	-
	<b>F clone 8.1</b>	+++	+++	++	++
<b>pTM1.128</b>	<b>G clone 8.2</b>	-/+	+	++	-
	<b>F clone 5.2</b>	+++	+++	++	++
<b>VTF7.3 infected HeLa (not transfected)</b>		-/+	+	+	-
<b>HeLa cells (not infected or transfected)</b>		-/+	+	+	-

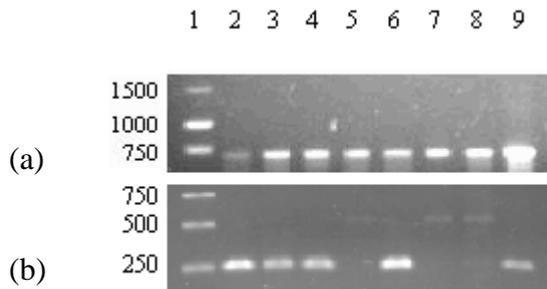
- No antibody staining
- + Small number of cells with a small amount of antibody staining
- ++ Approximately 50% of cells with specific antibody staining
- +++ Approximately 80% or more cells with specific antibody staining
- ++++ Approximately 100% or more cells with specific antibody staining

#### **4.6 Production of vaccinia virus recombinants**

Vaccinia virus clones were produced by initially infecting CV-1 cells with wild type vaccinia virus and transfecting them with either a maxiprep of pTM1.174F clone 6.1, pTM1.174G clone 3.6, pTM1.128F clone 5.2 or pTM1.128G clone 8.2 (Section 3.5.13). Lysates of these transfected CV-1 cells were then inoculated onto TK-143 cells under BuDR selection pressure to select for TK- recombinant virus and overlaid with agarose and neutral red to allow virus plaques to become visible (Section 3.5.14). Several individual plaques were collected for each co-infection and subsequently cultured in a 24 well plate containing TK-143 cells to produce a stock for DNA preparation (Section 3.5.15).

DNA was purified from vaccinia virus clones from both HMPV174 and HMPV128 F and G glycoprotein co-infections and amplified by PCR. DNA from vv174F clones 1

to 7 was amplified using primer set 1 (Table 3.8) under their optimised conditions (Table 3.9 and 3.12) whilst DNA from vv174G clones 1 to 7 was amplified using primer set 2 (Table 3.8) under the conditions described in NHOT (Table 3.9 and 3.12). The amplicand was resolved on a 1% agarose gel and visualised with ethidium bromide using ultraviolet light. The results are presented in Figure 4.20.

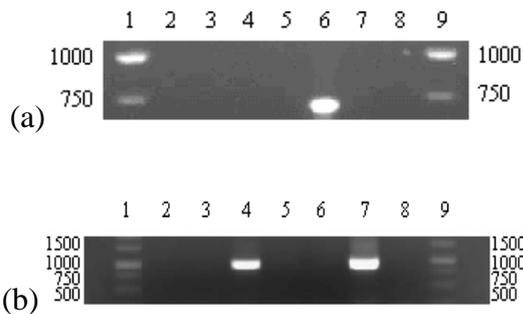


**Figure 4.20 PCR products of vv174F DNA preps and vv174G DNA preps**

Lanes are as follows; 1 – 1Kb molecular marker, 2a to 8a - vv174F clones 1 to 7, 2b to 8b – vv174G clones 1 to 7, 9a – pTM1.174F maxiprep at dilution  $10^{-3}$ , 9b - pTM1.174G maxiprep at dilution  $10^{-3}$  positive control.

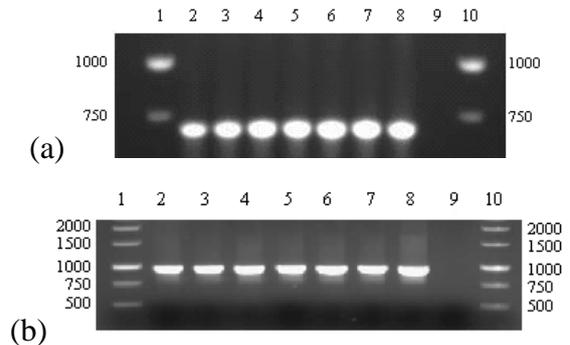
All vaccinia virus plaques derived from pTM1.174F clone 6.1 produced a band of around 700bp (Figure 4.20a) corresponding to the expected product size of 696bp. Four of the seven clones derived from pTM1.174G clone 3.6 produced a band of approximately 250bp corresponding to that expected for the primer set (Figure 4.20b). A band of 500bp can be observed in the other three clones. The vaccinia virus genome of these clones may contain a double insert as this length is twice the size of the expected product.

DNA from vv128F clones 1 to 7 was amplified using primer set 1 (Table 3.8) under their optimised conditions (Table 3.9 and 3.12) whilst PCR for vv128G clones 1 to 7 was performed using primer set 2 (Table 3.6) under the AliT2 conditions described in tables 3.9 and 3.12. The amplicand was resolved on a 1% agarose gel and visualised with ethidium bromide using ultraviolet light. The results are presented in Figure 4.21 and 4.22.



**Figure 4.21 PCR products of vv128F DNA preps and vv128G DNA preps**

Lanes are as follows; 1 – 1Kb molecular marker, 2a to 7a – vv128F clones, 2b to 7b – vv128G clones, 8 – negative PCR control, 9 – 1Kb molecular marker



**Figure 4.22 PCR products of recloned vv128F clone 5 and recloned vv128G clone 3 DNA preps**

Lanes are as follows; 1 – 1Kb molecular marker, 2a to 7a – vv128F clone 5 clones, 2b to 7b – vv128G clone 3 clones, 8a – pTM1.128F maxiprep at dilution  $10^{-3}$ , 8b – pTM1.128G maxiprep at dilution  $10^{-3}$ , 9 – negative PCR control, 10 – 1Kb molecular marker

Only one out of seven of the vaccinia virus recombinants derived from pTM1.128F clone 5.2 produced a band at 696bp with primers MF1499F and MF2175R (Figure 4.21a). However, after recloning the single positive plaque (vv128F clone 5) on TK-143 cells, all plaques picked contained the gene of interest (Figure 4.22a). Similarly, only two of the recombinants derived from pTM1.128G clone 2.3 produced a band of 901bp with the primer set used (Figure 4.21b) Plaque 3 was recloned and all derivative plaques contained the G insert (Figure 4.22b).

#### 4.7 Co-expression of HPMV vaccinia recombinants

All recombinant vaccinia virus clones (vv174F clone 7, vv128F clone 5.5, vv174G clone 5 and vv128G clone 3.5) were grown up to high titres by successive passage in L cells as described in section 3.5.15. Once passaged into 75cm<sup>3</sup> tissue culture flasks, each clone was titred according the method described in section 3.5.11.

Each clone was coinfectd into L cells with VTF7.3 at an MOI of 3 (Section 3.5.17). The infected cells were incubated for 24 hours, where after, the cells were scraped into PBS and either made into spot slides (Section 3.5.4) or inactivated for future use (Section 3.5.18).

The slides were tested for expression of the F and G antigen by immunofluorescence staining with both anti HMPV F glycoprotein, MAb24, and homologous and heterologous mouse polyclonal anti-HMPV serum (see Table 4.10 and Figure 4.23 and 4.24).

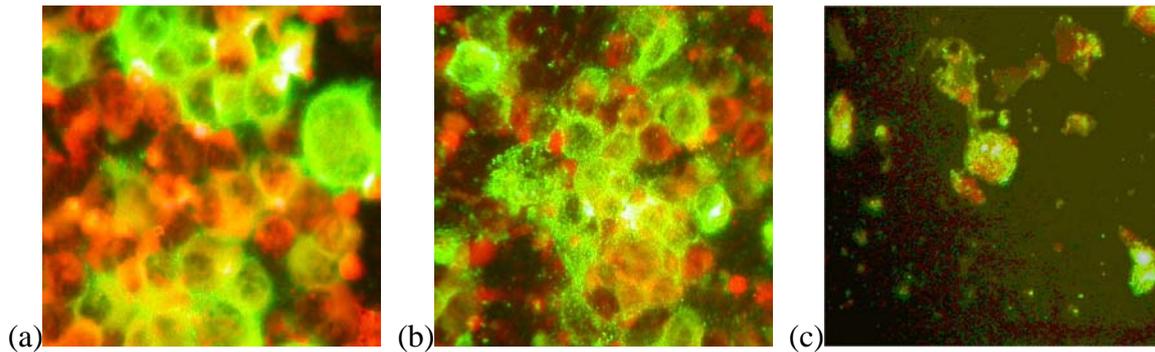
#### 4.8 Inactivation of vaccinia virus infectivity retaining HMPV antigenicity

Once coinfection was complete, cells were scraped into PBS and added to BEI for inactivation of the vaccinia virus over a 24 hour time period (Section 3.5.18). Treated virus was passaged three times, blind, in Vero cells as described in section 3.5.10 to ensure the vaccinia virus was fully inactivated. Immunofluorescence staining was repeated once the clones had been inactivated to ensure the antigenicity was preserved (Table 4.10 and Figure 4.23 and 4.24).

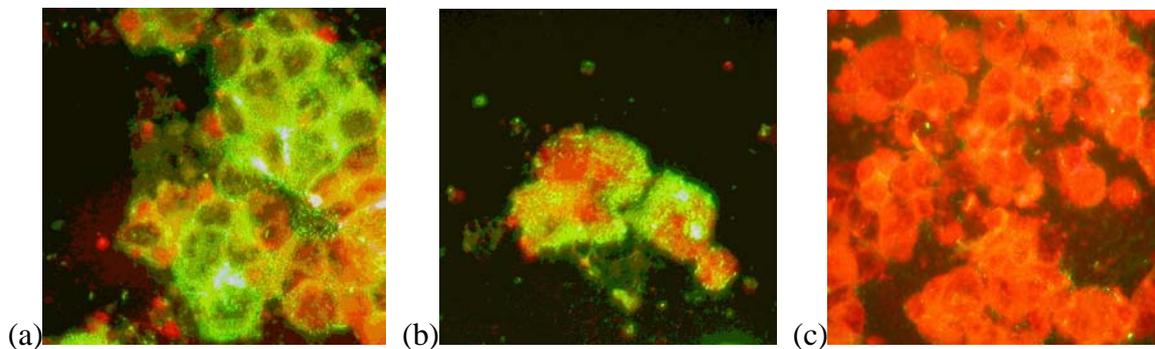
**Table 4.10 Reactivity of mouse anti-HMPV polyclonal serum with HMPV F and G genes from HMPV174 and 128.**

Virus (before / after inactivation)	Mouse polyclonal anti-HMPV antibody			MAb24
	Anti-174	Anti-145	Anti-128	
<b>vv174G5/VTF7.3</b>	+++ / +++	-/+ / -/+	-/+ / -/+	- / -
<b>vv174F7/VTF7.3</b>	++++ / +++	+++ / ++	++ / ++	++ / -
<b>vv128G3.5/VTF7.3</b>	-/+ / -/+	-/+ / -/+	++ / ++	- / -
<b>vv128F5.5/VTF7.3</b>	+++ / ++	+++ / ++	++ / ++	++ / ++
<b>VTF7.3 infected HeLa</b>	-/+ / -/+	-/+ / -/+	-/+ / -/+	- / -
<b>HeLa negative cells</b>	-/+ / -/+	-/+ / -/+	-/+ / -/+	- / -

- No antibody staining
- + Small number of cells with a small amount of antibody staining
- ++ Approximately 50% of cells with specific antibody staining
- +++ Approximately 80% or more cells with specific antibody staining
- ++++ Approximately 100% or more cells with specific antibody staining



**Figure 4.23** Example of immunofluorescence staining of L cells (a) coinfected with vv174F7 and VTF7.3 stained with MAb24 (b) coinfected with vv174F7 and VTF7.3 stained with anti-HMPV174 polyclonal antibody and (c) coinfected with vv174F7 and VTF7.3 after inactivation stained with anti-HMPV174 polyclonal antibody.



**Figure 4.24** Example of immunofluorescence staining of L cells (a) coinfected with vv174G5 and VTF7.3 (b) coinfected with vv174G5 and VTF7.3 after inactivation and (c) VTF7.3 only (before inactivation), all stained with anti-HMPV174 polyclonal antibody.

This technique allows the recombinant clones to be brought out of the designated lab for use in the development of glycoprotein specific antibody assays and to be used as a potential inoculum for animal work.

Once inactivated, the relative intensity of fluorescent staining in all clones was, to some extent, reduced with the mouse polyclonal anti-HMPV antibodies. The control VTF7.3 infected L cells displayed the same low levels of non-specific staining before and after inactivation. The MAb24 epitope was completely diminished in the inactivated vv174F/VTF7.3 clone 7 but surprisingly, this epitope was preserved in the vv128F/VTF7.3 clone 5.5. This result appeared robust as it was reproduced in three independent experiments.

## 4.9 Preparation of mono-specific mouse anti-sera

Four groups of three BALB/c mice were inoculated subcutaneously (Section 3.10.2) every two weeks with BEI inactivated preparations of vv174F7/VTF7.3, vv174G5/VTF7.3, vv128F5.5/VTF7.3 and vv128G3.5/VTF7 (Section 4.7). Thirty-five days after the initial subcutaneous inoculation, the serum antibody titres (Table 4.11) were established by titrating the final cardiac puncture by indirect immunofluorescence staining on homologous HMPV grown in 16HBE140 cells (see Section 3.5.4). Mice immunised with vv174G5/VTF7.3 and vv128G3.5/VTF7.3 received an additional subcutaneous inoculation on day 42 to boost serum antibody titres.

**Table 4.11 Antibody titres against the homologous strains of HMPV of the final cardiac puncture from vaccinia recombinant immunised mice.**

Inoculum	Titre (log <sub>10</sub> )
vv174F7/VTF7.3	3.11
vv174G5/VTF7.3	2.81
vv128F5.5/VTF7.3	3.11
vv128G3.5/VTF7.3	2.51

## 4.10 Strain specificity of mono-specific anti-sera by;

### 4.10.1 Immunofluorescence

Monospecific sera to vv174F7/VTF7.3, vv174G5/VTF7.3, vv128F5.5/VTF7.3 and vv128G3.5/VTF7.3 were diluted to 1/20 in PBS and tested by immunofluorescence on HMPV174, 145 and 128 infected 16HBE140 cells (Section 3.5.4). The results are presented in Table 4.12 and Figure 4.25.

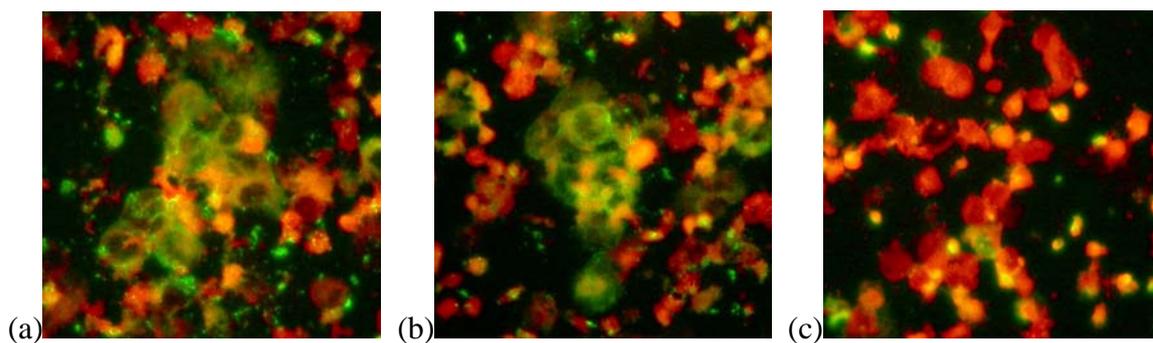
Monospecific anti-F174 and anti-F128 antibody preparations were cross reactive with all strains of HMPV tested. Surprisingly anti-G128 antibodies were cross reactive with both HMPV174 and HMPV145, however the staining pattern was less intense compared to both anti-F antibody preparations. Furthermore, anti-G174 antibodies were also slightly cross reactive with both HMPV128 and HMPV145 since the

staining pattern / intensity was above that observed in the negative controls. Whilst staining of HMPV infected 16HBE140 cells with HMPV anti-serum produces an apple green fluorescence which coats the cell, the anti-VTF7.3 serum produced a yellow strand like staining pattern which could be easily interpreted as non-specific staining.

**Table 4.12. Cross reactivity of monospecific anti-HMPV mouse serum on HMPV infected 16HBE140 cell cultures.**

Virus	Anti-174 G	Anti-174 F	Anti-128 G	Anti-128 F	Anti-VTF7.3
<b>HMPV174</b>	++++	++++	++	++++	<+
<b>HMPV145</b>	+	++++	+ / ++	++++	<+
<b>HMPV128</b>	+	++++	+++	++++	<+
<b>Uninfected 16HBE140 cells</b>	-	-	-	-	-

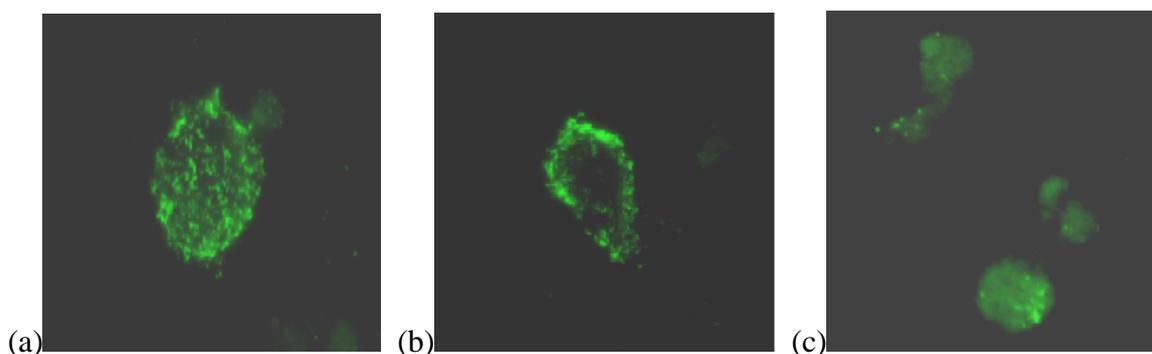
- No antibody staining
- + Small number of cells with a small amount of antibody staining
- ++ Approximately 50% of cells with specific antibody staining
- +++ Approximately 80% or more cells with specific antibody staining
- ++++ Approximately 100% or more cells with specific antibody staining



**Figure 4.25 Example of immunofluorescence staining of 16HBE140 cells (a) infected with HMPV128 (b) infected with HMPV174 and (c) uninfected all stained with monospecific anti-vv128G3.5/VTF7.3 antibodies.**

To ensure all antibody preparations were targeting the surface glycoproteins of HMPV, the serum samples were tested using membrane immunofluorescence of

unfixed HMPV infected and control 16HBE140 cells (Section 3.5.5). All antibodies produced specific membrane staining of cells infected with the homologous virus as seen in Figure 4.26, whilst the uninfected cells showed no sign of specific fluorescence with any of the monospecific antibodies.



**Figure 4.26. Example of membrane immunofluorescence staining in (a) HMPV174 infected cells stained with monospecific anti-vv174G5/VTF7.3 antibodies, (b) HMPV174 infected cells stained with monospecific anti-vv174F7/VTF7.3 antibodies and (c) uninfected 16HBE140 cells stained with monospecific anti-vv174F7/VTF7.3 antibodies.**

#### **4.10.2 Neutralisation**

HMPV174 and 128 infected 16HBE140 cell lysates predetermined to generate 75% of cells exhibiting specific antibody staining at day 15 were incubated with each monospecific antibody to produce a final dilution series of 1/10 to 1/2560 and inoculated onto 16HBE140 cells as described in section 3.5.8. At 15 days post-infection, spots were made and tested by immunofluorescence using HMPV monoclonal antibody pool (Section 3.5.4).

Each spot was rated by the number of infected cells together with the intensity of fluorescence in comparison with the positive control as described in Table 4.12. The cut off for neutralisation was < ++ (where the positive control was +++ ) and 50% neutralisation titres were calculated according to the Reed and Muench method. The results are displayed in Table 4.13.

**Table 4.13 Neutralisation of HMPV with monospecific antibodies**

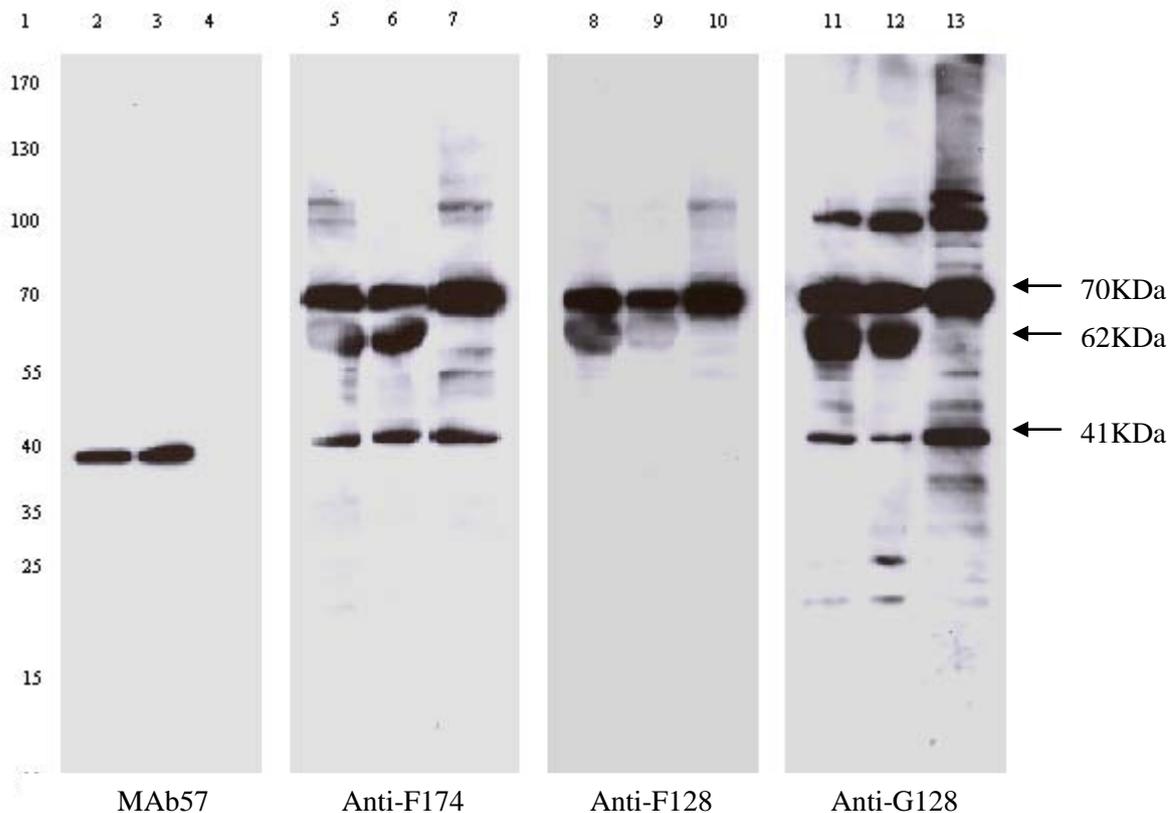
Monospecific antibody	50% serum neutralising antibody titre	
	(log <sub>10</sub> )	
	HMPV174	HMPV128
<b>α vv174F7/VTF7.3</b>	2.5	2.8
<b>α vv174G5/VTF7.3</b>	2.6	1.87
<b>α vv128F5.5/VTF7.3</b>	2.5	3.25
<b>α vv128G3.5/VTF7.3</b>	3.25	2.8
<b>α VTF7.3</b>	1.75	1.75
<b>Normal mouse serum</b>	≤ 0.7	≤ 0.7

Whilst the normal mouse serum had no neutralising activity, the anti-VTF7.3 serum neutralised both HMPV174 and HMPV128 to a titre of 10<sup>1.75</sup> suggesting the involvement of some sort of non-specific binding. However, both anti-F antibodies neutralised both strains to much higher titres indicating these antibodies have a higher neutralising capacity for HMPV than anti-vaccinia virus antibodies alone. This was also the case for anti-G antibodies where both antibody preparations neutralised the homologous strain of HMPV to a high titre. Surprisingly, whilst anti-G174 displayed similar neutralising ability to anti-VTF7.3 with HMPV128, anti-G128 antibodies also neutralised HMPV174 to a high titre (10<sup>3.25</sup>).

#### 4.10.3 Western blot

To further characterise the monospecific antibodies, uninfected 16HBE140 cells plus HMPV174 and 128 infected cultures were harvested after twelve days (Section 3.5.1) where 75% infectivity was confirmed by immunofluorescence (Section 3.5.4). These cultures were boiled in SDS reducing buffer before being loaded into a 3% to 18% gradient polyacrylamide gel and run overnight alongside molecular weight markers (Fermentas). The protein bands within the polyacrylamide gel were blotted onto PVDF membrane and stained with either a 1/500 dilution of monospecific antibody or MAb 57 at 5µg/ml. Antibody labelled bands were visualised with the secondary goat anti-mouse peroxidase conjugated antibody and the ECL chemiluminescence kit. To visualise the stained bands, membranes were exposed to photosensitive film which was then developed (see Section 3.23).

The resulting image (Figure 4.27) showed MAb 57 stained a strong band at 39KDa in both HMPV174 and 128 lanes, which was specific to HMPV and is consistent with the staining of the N protein using this protocol (Ingram, 2006 and G.L. Toms, personal communication). Monospecific anti – G174 blotted non-specifically both HMPV174 and 128 antigen and was thus uninterpretable despite being highly reactive in immunofluorescence (data not shown). All other monospecific antibodies were extremely cross reactive with all samples blotting two very heavy bands at 70KDa and 41KDa, with numerous heavier and lighter bands. All three antibodies also blotted a major band at approximately 62KDa in the HMPV positive lanes but there was no differentiation between anti - F and anti - G staining of either HMPV antigen.



**Figure 4.27. Comparison of HMPV174, 128 infected and uninfected 16HBE140 cell lysate stained with MAb57 and anti-HMPV monospecific antibodies in western blots.**

Lanes were loaded as follows; 1 – molecular weight marker (KDa); 2, 5, 8, 11 – HMPV174 infected cell lysate; 3, 6, 9, 12 – HMPV128 infected cell lysate; 4, 7, 10, 13 – uninfected 16HBE140 cells.

Text below the figure indicates antibody used to stain each blot. These results are representative of two independent experiments. \* The apparent HMPV128 specific band in lane 12 at 25KDa is observed in lanes 11 and 13 but is not visible in figure.

## Chapter 5: Hybridomas

Identification of hybridomas requires a sensitive and specific assay. ELISA offers sensitivity and a convenient format for rapidly screening a large number of hybridomas. However, conventional ELISA antigens contain cell culture reagents and host cell material and are not sufficiently specific when assaying for hybridomas generated from mice immunised with cell culture derived virus. By producing the recombinant protein in a cell line distinct from that used to grow the immunogen and by semi-purifying the recombinant glycoproteins on Concanavalin A, the specificity should be improved to a point where antigen specific hybridomas could be recognised.

The G glycoprotein expressed from vaccinia virus is fully glycosylated (Ball *et al.*, 1986) and unwanted cell culture antigens can be removed by lectin based chromatography using Concanavalin A, a lectin derived from the Jack Bean (Von Damme *et al.*, 1998), which binds to sugars found on RSV glycoproteins (Hendricks *et al.*, 1988). A more convenient method is to incorporate the lectin semi-purification into the ELISA assay. In the Concanavalin A capture assay ELISA plates are coated with the lectin to capture out un-purified G glycoprotein produced from recombinant vaccinia virus. This has been used successfully with both HIV and RSV glycoproteins (Robinson *et al.*, 1990; Robinson, 2007).

### 5.1 Development of Concanavalin A assay

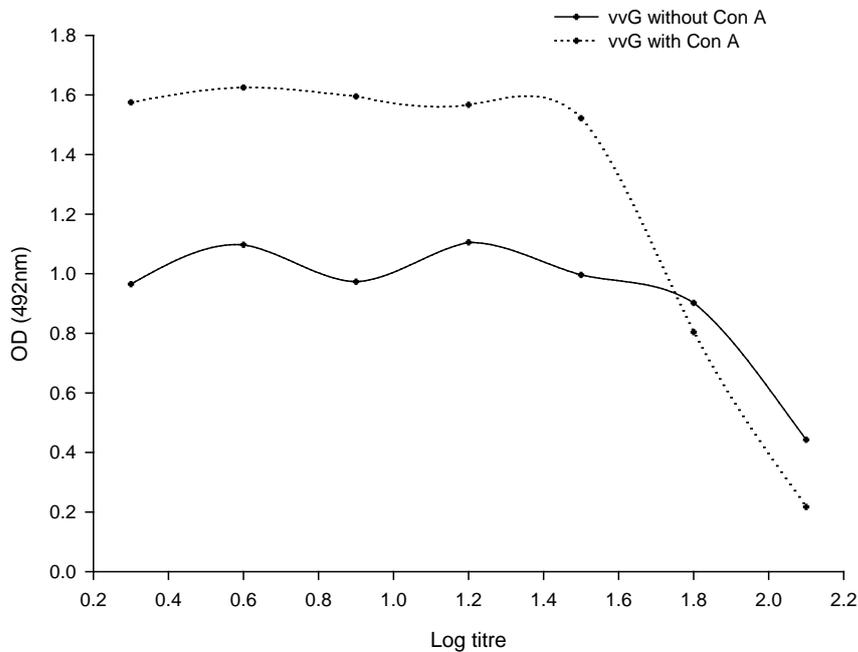
Initially, Concanavalin A capture of recombinant HMPV174 G glycoprotein expressed from a vaccinia virus grown in L cells was compared with the same antigen bound directly to the ELISA plate at pH 9.6.

Two ELISA plates coated with Concanavalin A were prepared as described in section 3.8.6. At the same time, another two ELISA plates were coated directly (50µl per well) with two-fold dilutions of recombinant vaccinia virus expressing HMPV174 G glycoprotein or VTF7.3 ELISA antigens (Section 3.8.1.2) from 1/2 to 1/128.

The next morning, the Concanavalin A coated plates were washed with PBSTx (Section 3.8.6) and 50µl per well dilutions of recombinant vaccinia virus expressing HMPV174 G glycoprotein and VTF7.3 ELISA antigens (Section 3.8.1.2) were bound

to the plates from 1/2 to 1/128 in PTF. These plates were incubated for 2 hours at 37°C. All plates were then washed with PBSTx and 25µl of polyclonal mouse anti – HMPV174 serum (Section 3.2.1.2) diluted 1/80 in PTF was added to each well and incubated for 1 hour at 37°C. The plate bound antibody was detected with GAMP for 1 hour followed by detection with OPD substrate (see Section 3.8.4 for methods).

The results are presented in Figure 5.1 and show Concanavalin A is capable and more efficient of capturing out vv174G inactivated antigen to produce a G specific ELISA without interference from the cell culture antigens.



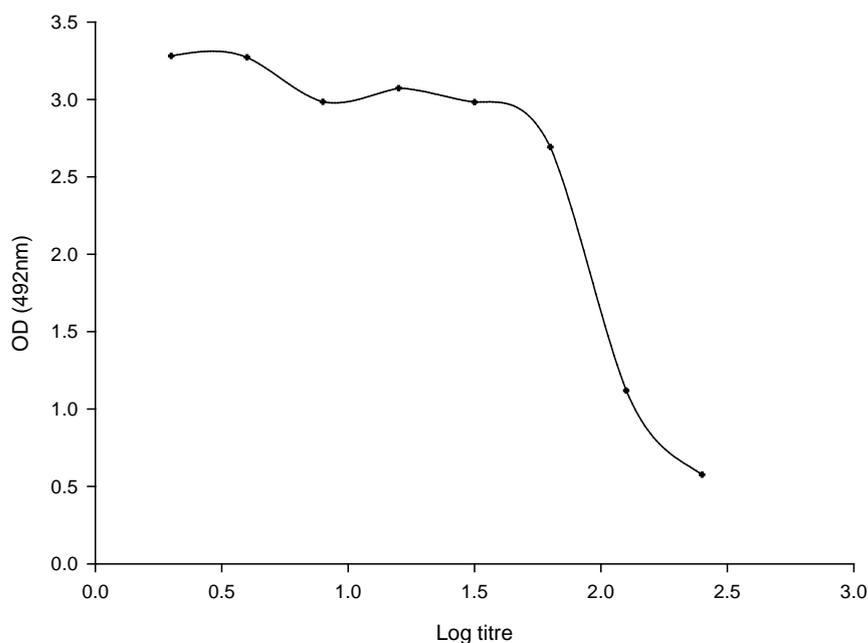
**Figure 5.1. A Concanavalin A capture and direct capture ELISA (subtracted values)**

## 5.2 Fusion using vaccinia virus Concanavalin A capture ELISA screen

Three mice were immunised with HMPV strain 174 (subtype A2) at a titre of  $5 \times 10^5$  ffu/ml according to the method described in section 3.11.

One mouse was bled one week after the last immunisation and the serum collected titrated on vv174G and VTF7.3 inactivated ELISA antigen using a Concanavalin A capture ELISA (Figure 5.2) (Section 3.8.6). 12 months post immunisation, the spleen

from the mouse was removed aseptically and splenocytes fused with NS1 cells as described in section 3.12. Blood collected at the time of splenectomy was titrated by immunofluorescence on HMPV174 infected 16HBE140 cells and gave a titre of 2.81 log<sub>10</sub>. Hybridomas were screened for subgroup specific anti-HMPV antibodies by Concanavalin A capture ELISA using vv174G and VTF7.3 antigens (Section 3.13.1). A total of 60 hybridoma vv174G positive wells were generated. Interestingly, during screening, several hybridomas produced high optical density readings with both vv174G and VTF7.3 antigen. As a result, all hybridomas that either had an optical density reading of at least 0.5 on both antigens, or a difference of more than 0.2 when comparing vv174G and VTF7.3 antigens, were picked for rescreening.



**Figure 5.2 Titration of HMPV174 immunised mouse serum on vv174G and VTF7.3 antigen (subtracted values)**

Rescreening by ELISA using HMPV174 antigen (Section 3.13.2) revealed 5 HMPV specific hybridomas that were cloned by limiting dilution as described in section 3.14. When retested by ELISA on HMPV174 infected HBE cell lysate and uninfected HBE cell lysate, four hybridomas were still producing subgroup specific MAbs and bound to HMPV174 antigen. These were MAbs AT1, 2, 4 and 5.

### 5.3 Characterisation of HMPV antibodies

#### 5.3.1 Determination of isotype and immunoglobulin concentration

For all characterisation experiments, MAbs were generated in 75cm<sup>3</sup> tissue culture flasks and allowed to overgrow. Antibody concentration was determined using control mouse serum in an isotype specific ELISA as described in section 3.8.4.

All MAbs were titrated simultaneously in all isotype specific ELISAs to simultaneously determine the isotype and immunoglobulin concentration. The results are presented in Table 5.1. None of the four MAbs reacted in the IgG2a assay (data not shown), MAbs AT1, 2 and 4 bound only to anti-IgG2b capture and MAb5 only to anti-IgG1 capture (Figure 5.3 and 5.4).

Table 5.1 MAb characteristics

MAb	Isotype	Ig concentration (µg/ml)
AT1	IgG2b	64
2	IgG2b	50
4	IgG2b	32
5	IgG1	14

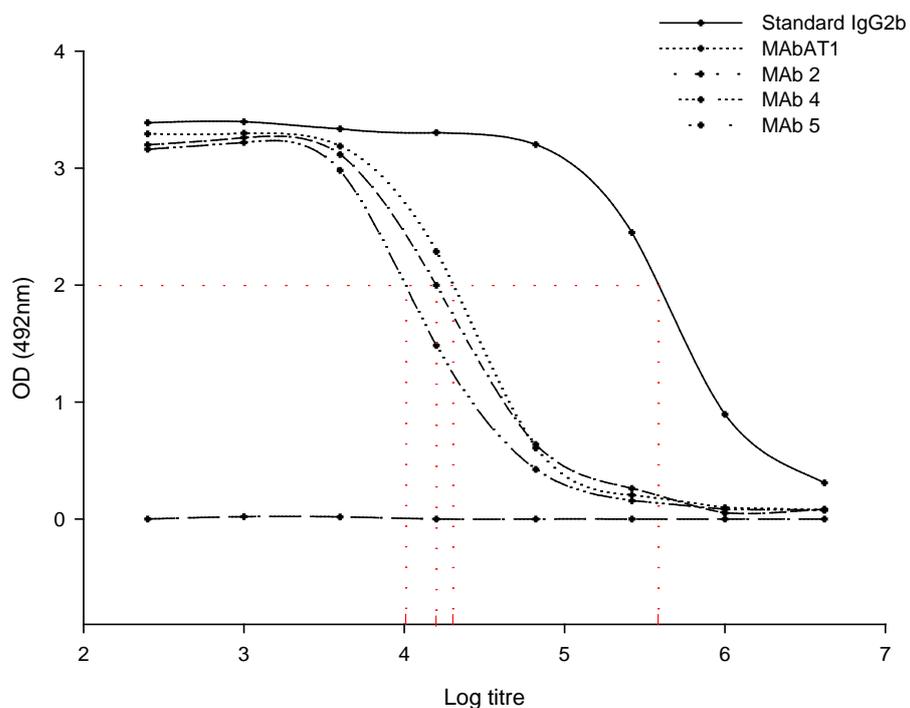
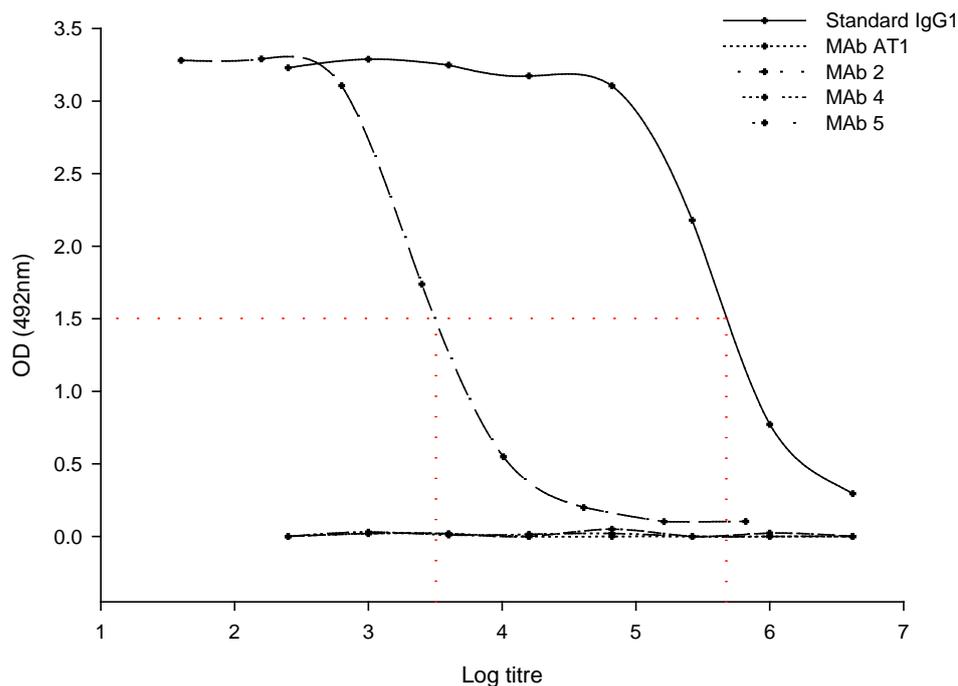


Figure 5.3 Titration of MAbs AT1, 2, 4 and 5 on IgG2b ELISA



**Figure 5.4 Titration of MAbAT1, 2, 4 and 5 on IgG1 ELISA**

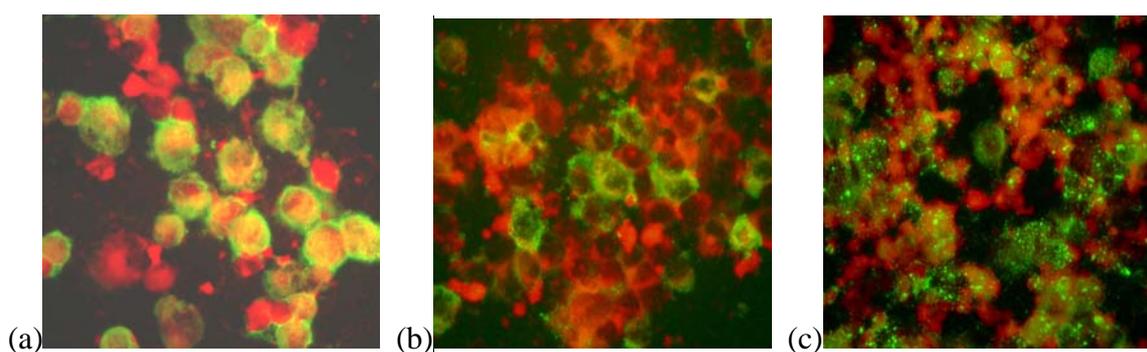
### 5.3.2 Immunofluorescence

To ensure the monoclonal antibodies were specific for the G glycoprotein, each MAb was tested for immunofluorescence staining of VTF7.3 infected HeLa cells transfected with pTM1.174F6.1, pTM1.174G3.6, pTM1.145F8.1, pTM1.145G2.3, pTM1.128F5.2 or pTM1.128G8.2 DNA (generated during the course of this study, see Section 4.3) as well as HMPV174, 145 and 128 infected 16HBE140 cells. The results are displayed in Table 5.2 and Figure 5.5.

Surprisingly, only MAbAT1 reacted with infected HeLa cells expressing HMPV174 G glycoprotein. MAbs 2 and 4 appear to be a strain specific anti F174 antibodies reacting with infected HeLa cells expressing HMPV174 F glycoprotein and HMPV174 only. MAb 5 appears to target an internal protein as this antibody has a distinct staining pattern, does not stain either F or G surface glycoproteins but is specific for HMPV174.

**Table 5.2 Reaction of monoclonal antibodies by immunofluorescence against HMPV174, 145 and 128 and transiently expressed HMPV F and G genes from HMPV174, 145 and 128.**

	Monoclonal antibody (titre log <sub>10</sub> )			
	AT1	2	4	5
HMPV174	2.81	2.51	2.51	2.51
HMPV145	-	-	-	-
HMPV128	-	-	-	-
pTM1.174 G clone 3.6	2.81	-	-	-
F clone 6.1	-	2.51	2.51	-
pTM1.145 G clone 2.3	-	-	-	-
F clone 8.1	-	-	-	-
pTM1.128 G clone 8.2	-	-	-	-
F clone 5.2	-	-	-	-
VTF7.3 infected HeLa (not transfected)	-	-	-	-
HeLa cells (not infected or transfected)	-	-	-	-
16HBE140 cells	-	-	-	-



**Figure 5.5 Example of immunofluorescence staining (a) in transiently expressed pTM1.174G3.6 with MAbAT1, (b) in transiently expressed pTM1.174F6.1 with MAb2 and (c) HMPV174 with MAb5.**

### 5.3.3 Neutralisations

HMPV174 infected 16HBE140 cell lysates were incubated with each monoclonal antibody to produce a final dilution series of 1/2 to 1/2048 and inoculated onto 16HBE140 cells as described in section 3.5.8. At 15 days post-infection, spots were made and tested by immunofluorescence using HMPV monoclonal antibody pool (Section 3.5.4).

Each spot was rated by the number of infected cells together with brightness of the fluorescence in comparison with the positive control and results are displayed in Table 5.3.

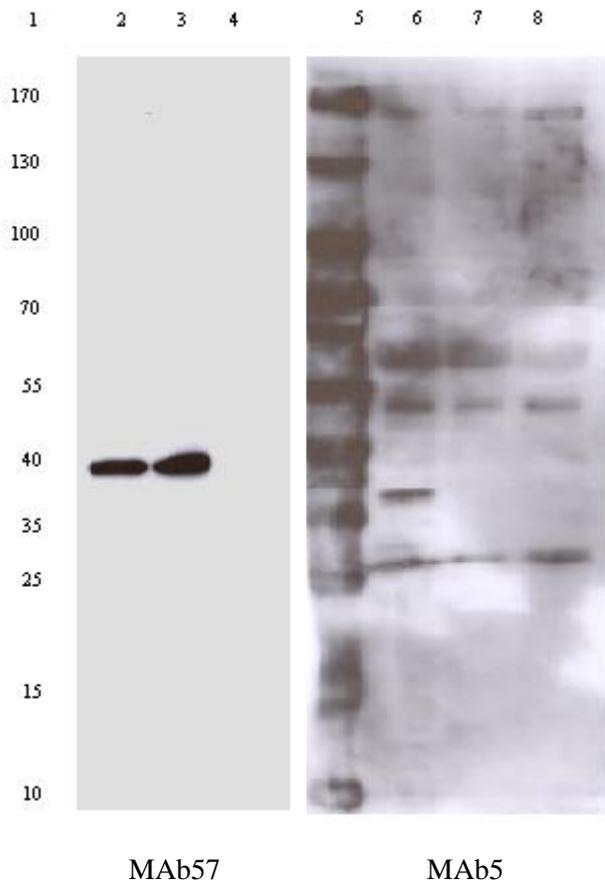
**Table 5.3 Neutralisation of HMPV174 with all monoclonal antibodies generated**

<b>Monoclonal antibody</b>	<b>50% serum neutralising antibody titre (log<sub>10</sub>)</b>	<b>50% inhibitory concentrations (µg/ml)</b>
<b>AT1</b>	≤ 0.3	> 40
<b>2</b>	0.45	17.7
<b>4</b>	2.41	0.13
<b>5</b>	≤ 0.3	7.01

Neither the anti-G specific MAb nor the unidentified MAb 5 neutralised the virus. Both anti-F MAbs however showed some neutralisation with MAb 4 more effective than MAb 2 despite their similar IgG concentration.

### 5.3.4 Western blot

Uninfected and infected HMPV cell cultures were prepared as before (Section 4.10.3) and run on a 3-18% gradient polyacrylamide gel overnight alongside molecular weight markers. The gel was blotted onto the PVDF membrane, which was subsequently stained with MAb57 at 5µg/ml and MAbs AT1, 2, 4 and 5 at 2µg/ml. MAb57 stained a 39KDa band, visible after only 30-second exposure. After an increased exposure time of 5 minutes, MAb5 stained a HMPV174 specific band at 38KDa, all other bands were cross reactive with both HMPV128 and 16HBE140 negative cells. MAbs AT1, 2 and 4 did not blot (data not shown).



**Figure 5.6 Comparison of HMPV174, 128 infected and uninfected 16HBE140 cells stained with either MAb57 or MAb5 western blot.**

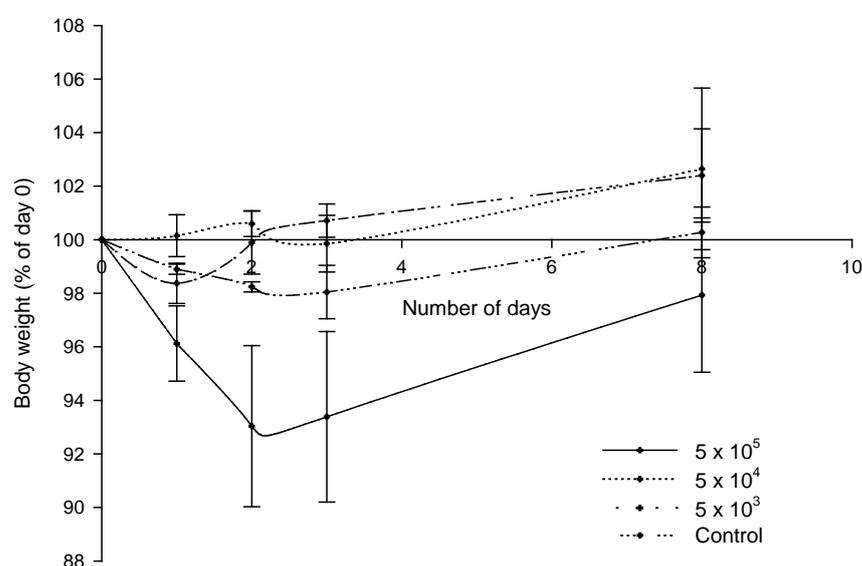
Lanes were as follows; 1, 5 – molecular weight markers; 2, 6 – HMPV174 infected cell lysate; 3, 7 – HMPV128 infected cell lysate; 4, 8 – uninfected 16HBE140 cells.

Text below the figure indicates antibody used to stain each blot. Blots are presented separately as a result of different exposure times.

## Chapter 6: Animal model

### 6.1 Establishing an animal model for HMPV

To determine the optimal titre at which to inoculate an animal model, where infection occurs but is not life threatening, four groups of three mice were inoculated intranasally with 10 fold serial dilutions of HMPV174 under isofluorane anaesthesia (Section 3.10.1). They were weighed daily to observe any alteration in weight post inoculation and the results are presented in Figure 6.1.



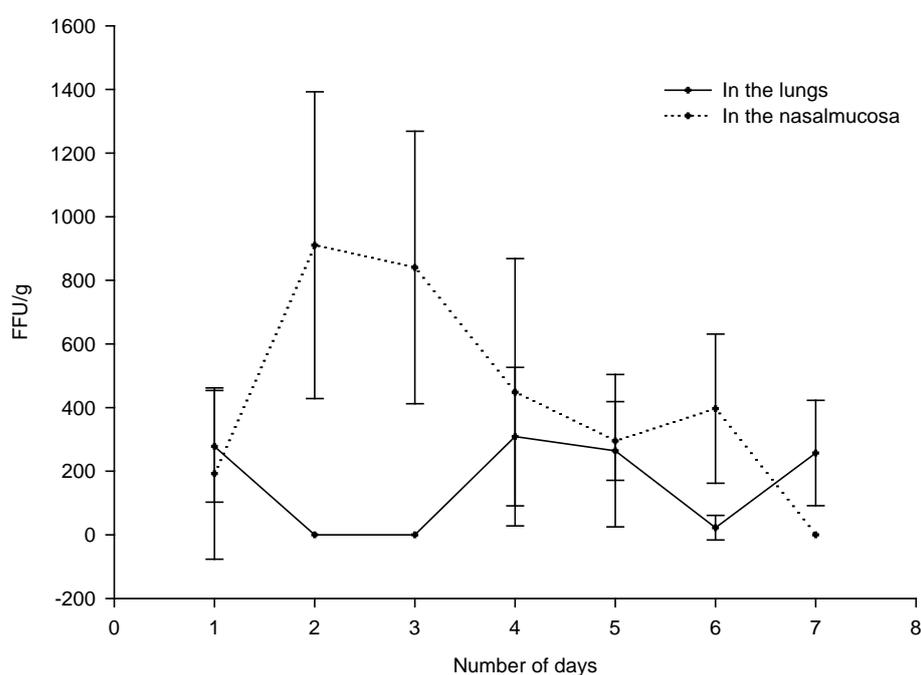
**Figure 6.1. Average weight increments of Balb/c mice inoculated with 10 fold serial dilutions of HMPV174**

The graph indicates that the mice who received a titre  $5 \times 10^5$  ffu/ml were the most distressed, determined by their slight loss in weight over the eight day period. The other groups appeared to have little or no response as their weights did not deviate and eventually increased towards the end of the week. Further experiments were carried out using the highest titre of HMPV174 as this was well tolerated by the animals.

## 6.2 Growth curve of HMPV in a mouse model

### 6.2.1 HMPV174 at $5 \times 10^5$ ffu/ml

To determine the stage during infection where the virus titre is at its highest *in vivo*, twenty one mice were intranasally inoculated (Section 3.10.1) with HMPV174 at  $5 \times 10^5$  ffu/ml (cell culture passage four, see Section 3.5.2). Each day post infection, three mice were killed, their lungs and nasal mucosa aseptically removed, homogenised and the supernatant assayed for infectivity of 16HBE140 cells (see Section 3.17 for methods).



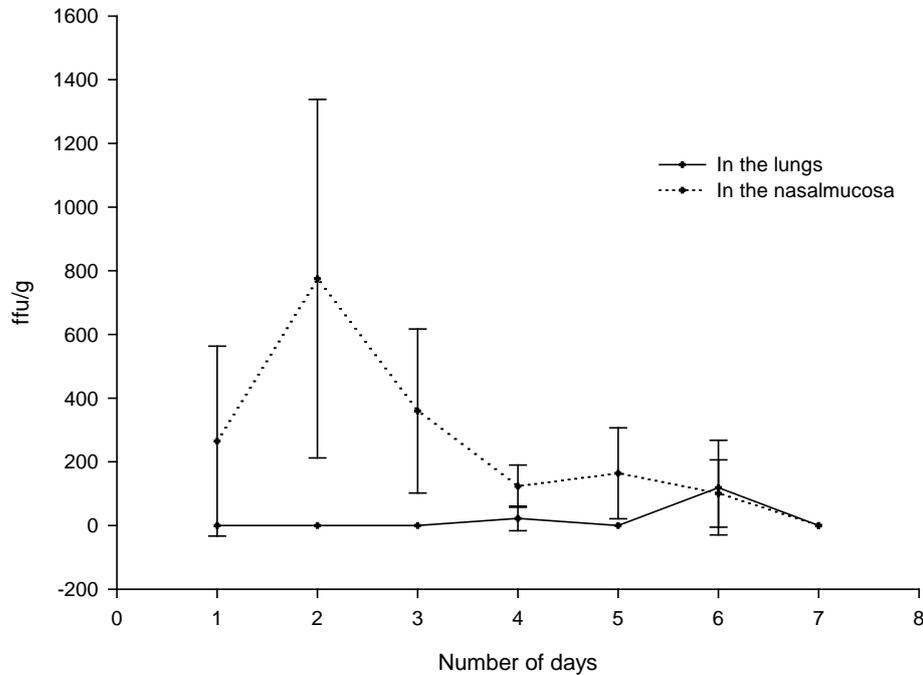
**Figure 6.2** Plaque forming units in the lungs and nasal mucosa of HMPV174 infected Balb/c mice.

The results are presented in Figure 6.2. In the nasal mucosa, the virus titre was at its highest two days post infection, peaking at  $10^{2.96}$  ffu/g, coinciding with maximum weight loss observed in Figure 6.1. In the lungs, titres were lower with a day 4 peak at  $10^{2.49}$  ffu/g.

### 6.2.2 HMPV174 at $2.6 \times 10^6$ ffu/ml

Since the previous experiment did not appear to elicit a substantial lower respiratory tract infection, HMPV174 was passaged several times on 16HBE140 cells as

previously described (Section 3.5.2) to increase the titre before repeating the above experiment. A titre of  $2.6 \times 10^6$  ffu/ml was achieved at passage 11, which was subsequently inoculated intranasally (Section 3.10.1) into another twenty one mice as before. The results are presented in Figure 6.3.



**Figure 6.3** Plaque forming units in the lungs and nasal mucosa of HMPV174 infected Balb/c mice.

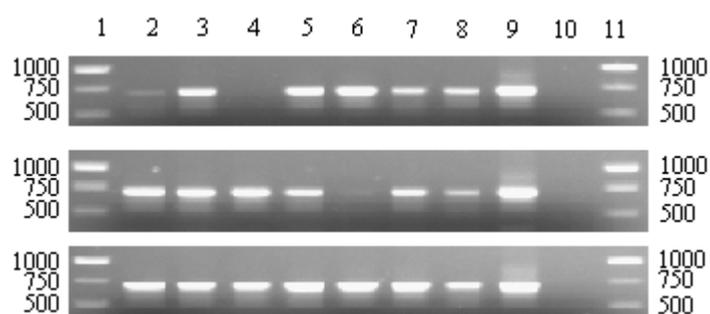
Again, virus was detected in the nasal mucosa at day two, albeit at slightly reduced titre, peaking at  $10^{2.89}$  ffu/g. However, virus was unable to establish an infection in the lower respiratory tract, with even lower yields being recovered than with the lower dose.

### 6.2.3 HMPV145 at $2 \times 10^6$ ffu/ml

To investigate whether the inability to retrieve infectious virus was attributable to the virus strain,  $2 \times 10^6$  ffu/ml of another strain, HMPV145 (passage 9 in 16HBE140 cells) was inoculated intranasally (Section 3.10.1) into another twenty one mice as before. The lungs and nasal mucosa from each mouse were aseptically removed, homogenised and the supernatant was infected onto 16HBE140 cells to calculate the titre using a fluorescent focus assay. The results revealed no infectious virions were present in any of the samples obtained by dissection

### 6.3 PCR

To ensure, HMPV174 was present in the samples recovered after dissection of mice inoculated with  $2.6 \times 10^6$  ffu/ml of HMPV174 (see Section 6.2.2), viral RNA was extracted from macerate supernatants using a QIAamp viral RNA minikit. The purified RNA was subsequently assayed in a reverse transcription PCR using primer set 1 (Table 3.8) and the F glycoprotein gene as a target (see Section 3.6 and Table 3.9 and 3.12 for conditions). The results displayed in Figure 6.4 confirm the presence of viral RNA in the homogenised samples of lung, and indicates the quantity of RNA increases over time to peak between day 4 and day 6.



**Figure 6.4 Amplification of F glycoprotein RNA extracted from lungs lysates obtained in the HMPV174  $2.6 \times 10^6$  growth curve.**

Lanes are as follows; 1 – 1Kb molecular marker, 2 to 8 – day 1 to day 7 post inoculation (each band represents one mouse), 9 – PCR positive control, 10 – PCR negative control, 11- 1Kb molecular marker

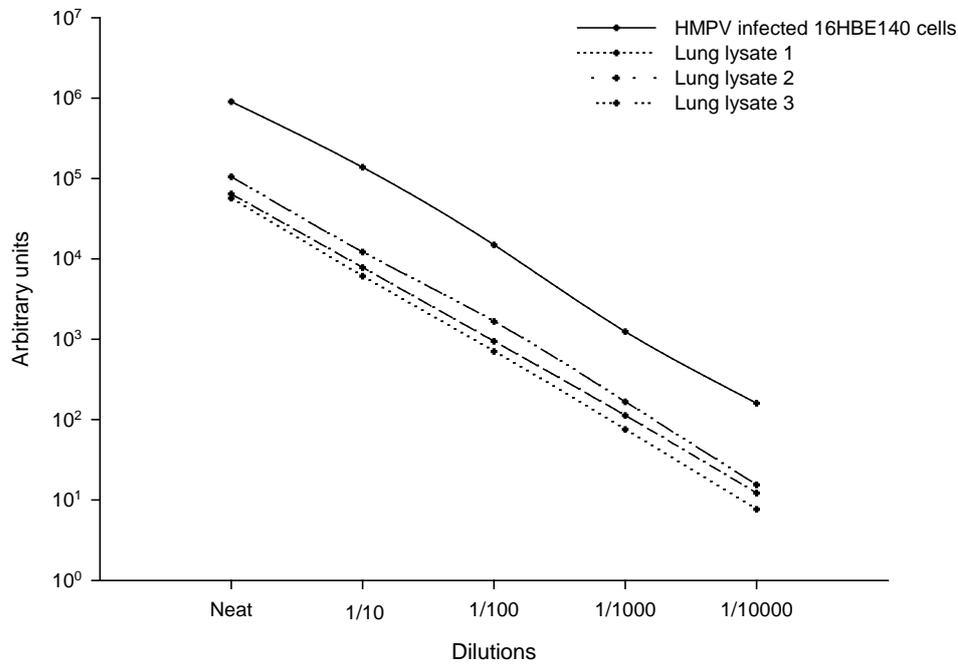
### 6.4 Quantitative PCR (qT-PCR)

To obtain quantitative results of the growth curve above, a qT-PCR assay was established using a method based on Maertzdorf *et al* (2004), where primers and probes were derived from the nucleoprotein gene. The N gene was chosen as a target because it is one of the most conserved genes in the HMPV genome. cDNA was synthesised as above using the N-LN primer set and conditions stated in Table 3.10, 3.11 and 3.12. The standard curve was constructed as described in section 3.6.8.1.

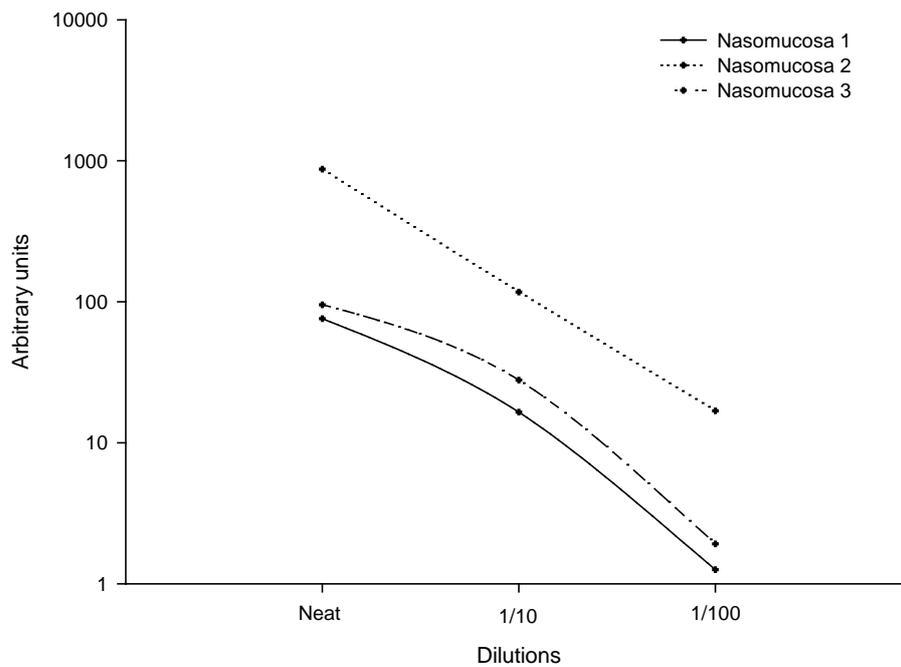
#### 6.4.1 Optimisation of the concentration of RNA template

In order to investigate the tissue macerates for the presence of inhibitors, quantitation was carried out on ten-fold dilutions of RNA extracted from the lung and nasal mucosa samples as well as viral RNA extracted from HMPV infected 16HBE140 cells. Dilutions were set up from neat to 1/10000 for lung lysates and 1/100 for nasal

mucosa samples using DEPC water plus carrier RNA. PCR was carried out in triplicate and the mean amount of RNA genome was determined for each dilution factor. The results are presented in Figure 6.5 and Figure 6.6.



**Figure 6.5. Optimisation of RNA from lung samples for qT-PCR.**



**Figure 6.6. Optimisation of RNA from nasal mucosa samples for qT-PCR.**

Figure 6.5 shows the amount of PCR product does not increase with the dilution of RNA from either the lung lysates or the HMPV infected cells observed by the linear plots. PCR product from the nasal mucosal RNA (Figure 6.6) revealed a slight increase at 1/10 dilution compared to the neat sample and would suggest some unknown PCR inhibitors may be present in the RNA samples. Therefore, as a precaution, in subsequent qT-PCR, all RNA templates derived from lung lysates were diluted to 1/100 and from nasal mucosal samples to 1/10 before addition to the reaction mix unless otherwise stated.

#### 6.4.2 HMPV174 $2.6 \times 10^6$ ffu/ml lung lysates

Viral RNA from the lung lysates was extracted as before and cDNA synthesised using the N-LN primer set as stated above. For quantitation, 6 $\mu$ l of cDNA was added to 24 $\mu$ l of reaction mix (Table 3.11) and run as stated in methods 3.6.8. Figure 6.7 illustrates that after day 3, when the inoculum was cleared by the host, an increase in viral RNA genome which peaks at  $10^{4.96}$  arbitrary units at day 4. This would suggest whilst most of the inoculated virus was cleared by the hosts immune system upon invasion, some virus was able to escape and begin infection before being detected and completely removed.

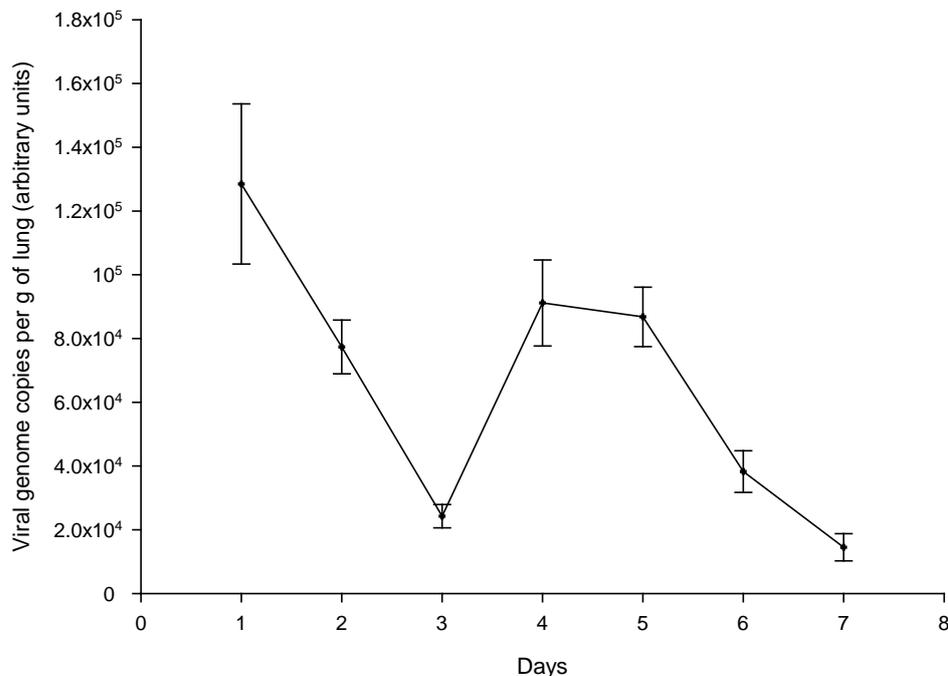
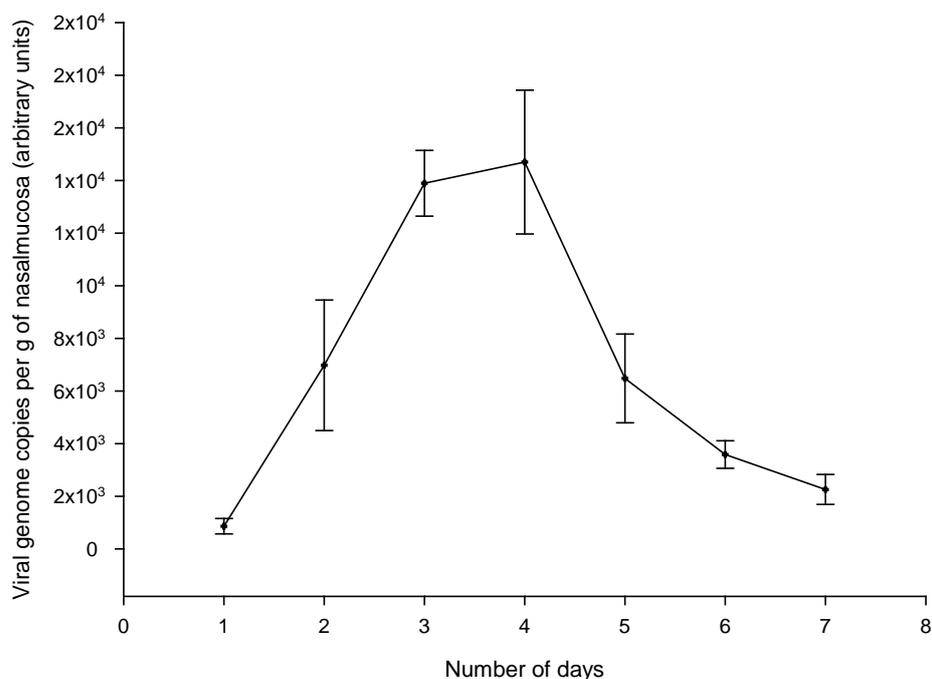


Figure 6.7 Average quantities of viral RNA genome extracted from lung lysates of the HMPV174  $2.6 \times 10^6$  ffu/ml growth curve.

### 6.4.3 HMPV174 $2.6 \times 10^6$ ffu/ml nasal mucosal samples

Viral RNA was extracted and cDNA prepared as above for all nasal mucosal samples from the growth curve. These were subsequently run in the qT-PCR assay to establish the relative levels of viral RNA genome.



**Figure 6.8** Average quantities of viral RNA genome extracted from nasal mucosa samples of the HMPV174  $2.6 \times 10^6$  ffu/ml growth curve.

Results presented in Figure 6.8 show an increase in viral RNA from day 1, peaking at  $10^{4.17}$  arbitrary units at day 4 before trailing off.

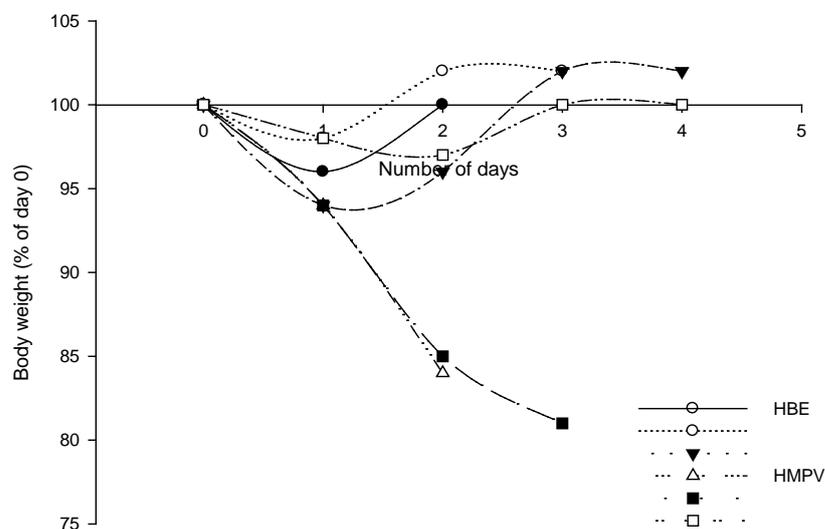
### 6.5 HMPV174 at $7 \times 10^6$ ffu/ml

Despite the presence of viral RNA in both the lungs and nasal mucosa of the mouse model, an inoculum of  $2.6 \times 10^6$  ffu/ml resulted in very low infectivity titres in the lungs and only trivial weight loss. To discover if an even higher titre of virus would result in higher levels of replication, HMPV174 was passaged once more in 16HBE140 cells (Section 3.5.1) and cells from 9 plates were harvested and resuspended in a total of 3ml of MM to produce a 9 fold increase in the virus

concentration. A titre of  $7 \times 10^6$  ffu/ml was achieved. This stock of HMPV was inoculated intranasally (Section 3.10.1) into three mice with another three mice receiving the same concentration of uninfected 16HBE140 cells as a negative control.

### 6.5.1 Weights post infection

Measurement of weight pre and post inoculation was essential to assess the health of the animals and was also an indicator of whether the animal had succumbed to infection.



**Figure 6.9** Weight of each animal post inoculation with HMPV at  $7 \times 10^6$  ffu/ml.

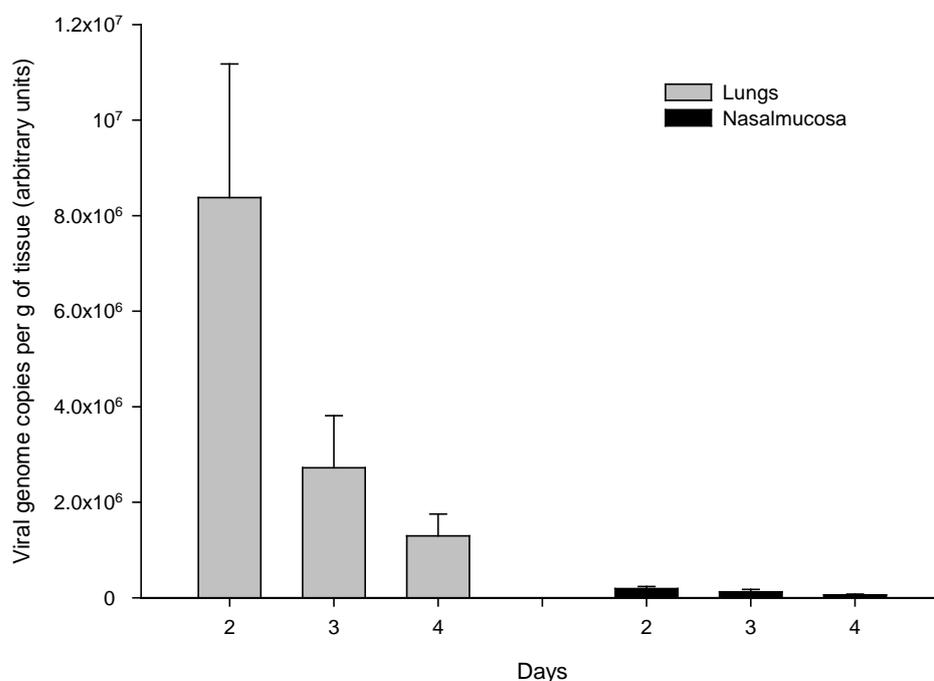
Two of the HMPV inoculate mice exhibited rapid weight loss evident from day 2 post infection. For the third infected mouse, weight loss was trivial and returned to pre-inoculation levels by day 3. For mice inoculated with uninfected cells, minor weight loss occurred 24 to 48 hours post inoculation returning to pre-inoculation levels by day three. Of the two mice infected with HMPV174, which showed severe weight loss two days post infection (Figure 6.9), one mouse was killed on day 2 and the other on day 3 (mice must be culled if they lost more than 20% of their original body weight). Two HBE infected mice were killed alongside these to act as negative controls. The last HMPV and HBE infected mice were killed on day 4.

### 6.5.2 Infectivity

The lungs and nasal mucosa from each mouse were aseptically removed, homogenised and the supernatant was infected onto 16HBE140 cells to calculate the titre using a fluorescent focus assay. The results revealed no infectious virions were present in any of the samples obtained by dissection.

### 6.5.3 QT-PCR

To ensure viral RNA was present in the samples, quantitative PCR was performed on the RNA extracted from all specimens as before. The results in Figure 6.10 show there to be a large amount of HMPV RNA present in all samples.

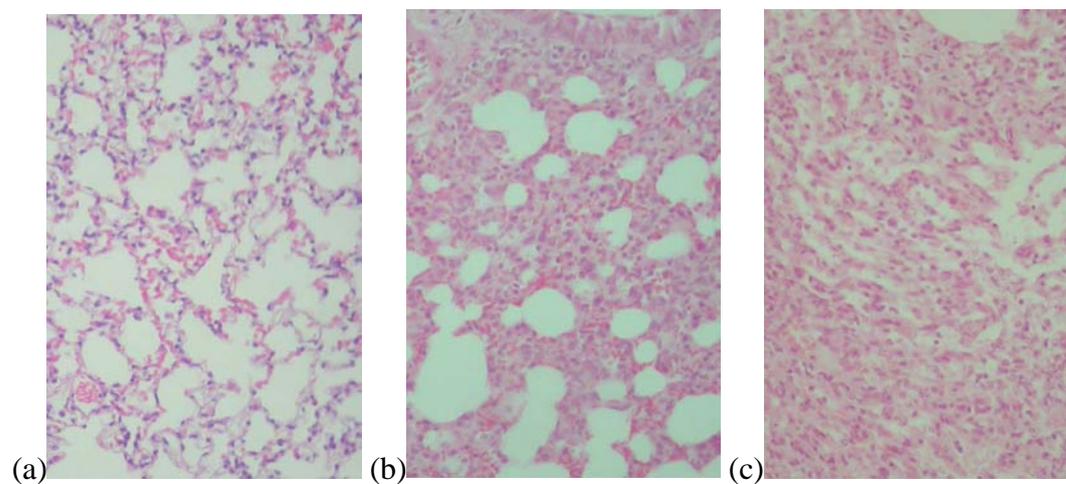


**Figure 6.10** Quantity of viral RNA present in the lungs and nasal mucosa of HMPV174 infected Balb/c mice.

## 6.6 Histochemistry and Immunohistochemistry

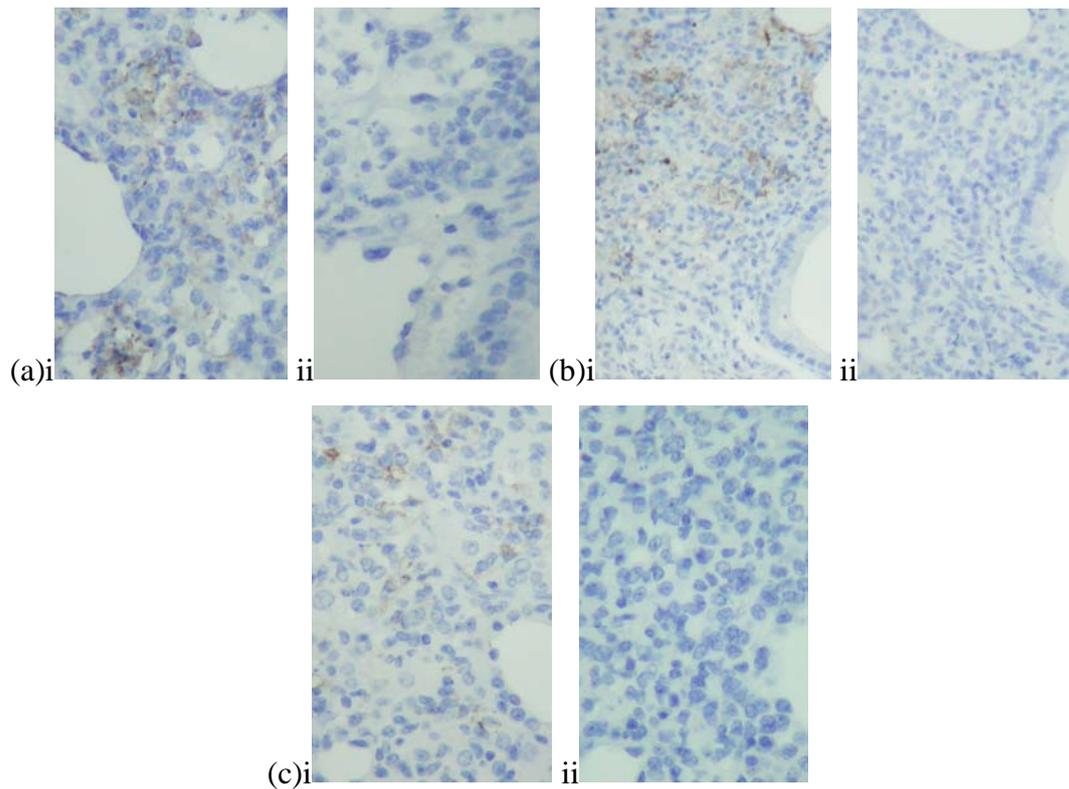
To obtain a better understanding of the damage occurring in the lungs of mice inoculated with  $7 \times 10^6$  ffu/ml of HMPV, formaldehyde fixed and wax embedded sections of mouse lung were stained with haematoxylin and eosin or with anti-HMPV

monoclonal antibody pool by immunohistochemistry (Section 3.19). Histological differences were observed between mice inoculated with HMPV174 and those immunised with 16HBE140 cells (Figure 6.11). Whilst uninfected, 16HBE140 cell inoculated mice showed low levels of peri-bronchiolar infiltration, lungs from HMPV infected mice had significant interstitial infiltration of inflammatory cells. There was evidence of large areas of consolidation together with destruction of alveolar walls which was markedly different from the sham infected mice.



**Figure 6.11 Haematoxylin-eosin staining of lungs (a) uninfected, (b) mock infected with 16HBE140 cells and (c) infected with HMPV174**

Immunolabelling of mouse lung stained with anti-HMPV monoclonal pool (Section 3.19) revealed there to be large areas of viral protein expression, mainly observed in the lungs of mice two and three days post-infection. There was extensive granular staining of the cytoplasm which was mainly located around the bronchioles (Figure 6.12).



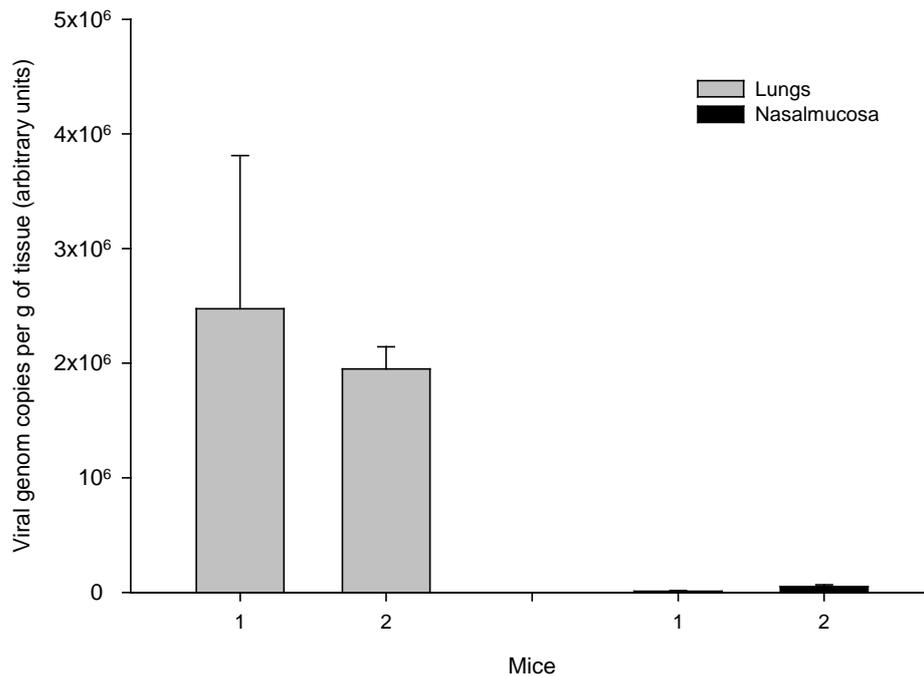
**Figure 6.12 Immunohistochemical staining for HMPV ((i) with and (ii) without primary antibody) in mice lungs (a) 2 days post-infection, (b) 3 days post-infection and (c) 4 days post-infection.**

### **6.7 Infectivity study with the addition of trypsin**

As both the quantitative PCR and immunohistochemistry results revealed evidence of HMPV RNA and antigen present within the lungs and nasal mucosa of infected mice, it suggests that RNA replication and transcription is occurring. Virus infectivity recovered is, however, minimal. Virus recovered from human tissues is incapable of replicating in some cell lines without the addition of exogenous protease. To test whether exogenous protease was required for infectivity of virus in mouse respiratory tissue macerates, two mice were inoculated intranasally with HMPV174 at a titre of  $2.6 \times 10^6$  ffu/ml. At 5 days post-infection, lungs and nasal mucosa were collected, homogenised in SAFMM with 0.007% trypsin before being inoculated onto 16HBE140 cells as previously described (Section 3.17).

Infectivity results revealed there to be no infectious virus present in the homogenised samples with or without the addition of trypsin.

Quantitative PCR was performed on the same samples to ensure HMPV was present in both the lungs and nasal mucosal dissections. The results confirm the presence of viral RNA in all samples and are presented in Figure 6.13.



**Figure 6.13** Quantity of viral RNA present in the lungs and nasal mucosa of HMPV infected mice after the addition of trypsin.

These experiments indicate that intranasal inoculation of 16HBE140 HMPV174 infected cell lysate results in infection of a substantial number of cells in the respiratory tract of the mouse with viral RNA replication and viral protein synthesis increasing to peak at day 3 post infection in the nasal mucosa and day 4 in the lungs. This is however, associated with only a low level of infectious virus in the nasal mucosa and even lower level in the lungs.

## 6.8 Challenge experiment

This experiment was designed to establish whether a series of immunisations with the G glycoprotein of HMPV174 would protect against challenge with the whole virus *in vivo*. Three groups of 6 mice were immunised by subcutaneous inoculations of antigen in Freund's adjuvant according to the schedule in section 3.18. The first group was immunised with inactivated vv174G5/VTF7.3 coinfecting L cells as prepared in section 4.7 and 4.8, another group with inactivated VTF7.3 infected L cells (Sections 4.7 and 4.8) and the final group with 16HBE140 cells infected with HMPV174 as described in section 3.5.1. To boost mucosal immunity, all groups were immunised intranasally with the same antigen as above prepared in cholera toxin (Section 3.10.1) to stimulate local IgA responses.

Once systemic immunity had been established, as judged by serum IgG antibody responses to virus infected cells (Table 6.1) by immunofluorescence, two mice from each group were sacrificed by cardiac puncture, blood was collected and their nasal mucosa washed with PBS. The rest of the mice were subsequently challenged with  $2.6 \times 10^6$  ffu/ml HMPV174 infected 16HBE140 cells by intranasal inoculation under isoflurane anaesthesia (Section 3.10.1) and their weights monitored over a five day period. On the fifth day, all mice were sacrificed by cervical dislocation and the lungs and nasal mucosa aseptically removed for infectivity studies (Section 3.17) and quantitative PCR assays (Section 3.6.8).

**Table 6.1 Antibody titres of tail bleeds from HMPV174, vv174G5/VTF7.3 and VTF7.3 immunised mice**

<b>Inoculum</b>	<b>Titrated on:</b>	<b>Titre (log<sub>10</sub>)</b>
<b>HMPV174</b>	vv174G5/VTF7.3 infected L cells	3.4
<b>vv174G5/VTF7.3</b>	HMPV174 infected 16HBE140 cells	3.1
<b>VTF7.3</b>	HMPV174 infected 16HBE140 cells	-

## **6.8.1 ELISA of serum and nasal-secretions pre-challenge**

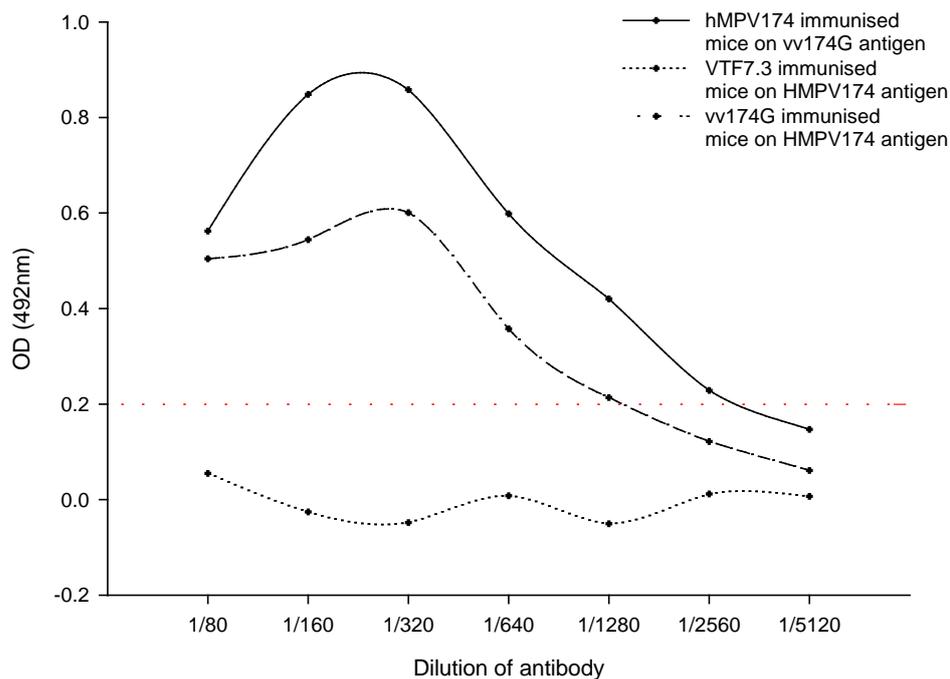
### **6.8.1.1 Serum antibody**

Serum was obtained from the blood taken by cardiac puncture from the pre-challenged mice and tested by ELISA for anti – IgG antibody (see Section 3.8), as this is the predominant antibody found in blood, on both whole HMPV virus antigen and on vv174G5/VTF7.3 and VTF7.3 inactivated antigen (Section 3.8.1) using the vaccinia virus capture assay as described in section 3.8.6.

An ELISA plate was coated with HMPV174 ELISA antigen on one half and 16HBE140 negative ELISA antigen on the other as described in section 3.8.5. At the same time, another plate was coated with Con A as previously described (Section 3.8.6). The next morning, the Con A plate was washed and charged with vv174G5/VTF7.3 and VTF7.3 ELISA antigens as described in section 3.8.6. Both plates were then washed and dilutions of the serum samples (1/80 to 1/5120) from the vv174G5/VTF7.3 and VTF7.3 immunised mice were incubated on the HMPV coated plate, whilst the HMPV174 immunised serum sample dilutions were charged to the vv174G and VTF7.3 coated plate. Both plates were also coated with HMPV polyclonal mouse serum as a positive control and incubated for 90 minutes, before being washed and bound antibody detected with NCL-GAMP at 1/1000 followed by OPD (see Section 3.8.4 for methods).

The results are shown in Figure 6.14. HMPV174 immunised mice had high levels of antibody directed towards vv174G5/VTF7.3 antigen. Similar results were seen in the vv174G5/VTF7.3 immunised mice, albeit slightly lower titres, when tested on HMPV174. However, no immunity to HMPV174 could be observed in the VTF7.3 immunised animals.

Assuming levels of IgG in the mice above are similar to the levels found in standard mouse serum (7-10mg/ml), at the end point dilution where the optical density is at 0.2, the concentration of antibody is ~355µg/ml for the HMPV immunised mice and ~162µg/ml for the vv174G5/VTF7.3 immunised mice.



**Figure 6.14 Serum samples obtained from pre-challenged mice titrated on HMPV174 infected 16HBE140 cells or recombinant vaccinia virus expressing HMPV174 G glycoprotein ELISA antigens.**

### 6.8.1.2 Nasal antibody

Intranasal inoculations with cholera toxin adjuvant were designed to stimulate the development of mucosal immunity pre-challenge. With IgA antibody being the predominant immunoglobulin in the mucosa, anti – HMPV IgA antibody in the nasal mucosal washes was measured by ELISA using the same method as the serum samples above (Section 6.7.1.1). This method proved unable to detect either mouse IgA or anti-viral IgA antibodies in these samples for reasons which have not been resolved.

### 6.8.2 Neutralisation

HMPV174 infected 16HBE140 cell lysates predetermined to generate 75% of cells exhibiting specific antibody staining at day 15 were incubated with each serum sample to produce a final dilution series of 1/10 to 1/2560 and inoculated onto

16HBE140 cells as described in section 3.5.8. At 15 days post-infection, spots were made and tested by immunofluorescence using HMPV monoclonal antibody pool (Section 3.5.4).

Each spot was rated by the number of infected cells together with the intensity of fluorescence in comparison with the positive control as described in Table 4.12. The cut off for neutralisation was < ++ (where the positive control was +++ ) and 50% neutralisation titres were calculated according to the Reed and Muench method. The results are displayed in Table 6.2.

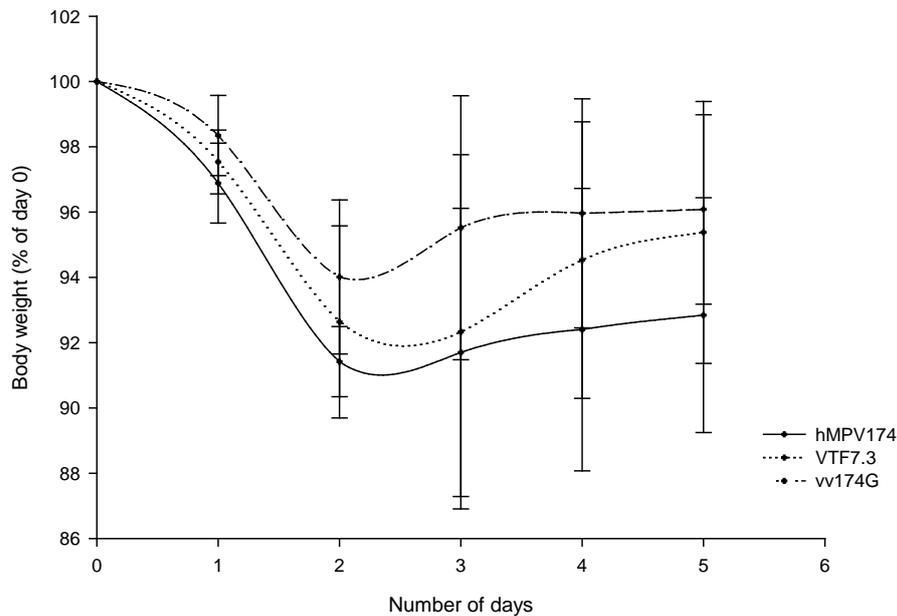
**Table 6.2 Neutralisation of HMPV174 with all three serum samples collected pre-challenge**

<b>Serum</b>	<b>50% serum neutralising antibody titre (log<sub>10</sub>)</b>
<b>HMPV174</b>	-
<b>vv174G5/VTF7.3</b>	2.62
<b>VTF7.3</b>	1.75

Whilst the anti-VTF7.3 serum neutralised HMPV174 to a titre of  $10^{1.75}$ , anti-G174 antibodies neutralised HMPV174 to a much higher titre indicating these antibodies have a higher neutralising capacity for HMPV than anti-vaccinia virus antibodies alone. The neutralising capacity of anti-HMPV174 antibodies could not be measured due to the high levels of anti-16HBE140 cell antibodies present in the preparation.

### **6.8.3 Weights post-challenge**

To monitor the health of the mice post-challenge, weights were taken each morning and the average weight loss of each group is shown in Figure 6.15. Surprisingly, the group immunised with HMPV174 lost the most amount of weight, almost 10%. A two-way ANOVA showed this weight loss was significantly different in comparison to mice immunised with vv174G5/VTF7.3 ( $P=0.01$ ,  $F=8.80$ ). However, there was no significant difference between the weight loss of HMPV174 and VTF7.3 infected mice.



**Figure 6.15 Average weight increments of Balb/c mice post-challenge with HMPV174.**

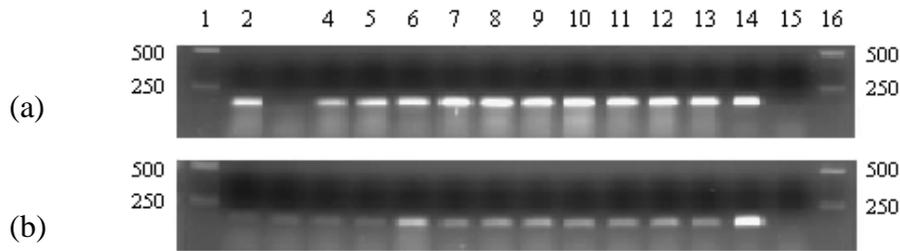
#### **6.8.4 Infectivity**

Samples extracted from the lungs and nasal mucosa were homogenised and the macerates titrated for infectivity onto 16HBE140 cells. No infectious virions were present in any of the samples collected.

#### **6.8.5 PCR**

To ensure, HMPV174 was present in the samples recovered after dissection, viral RNA was extracted from the supernatant using a QIAamp viral RNA minikit. The purified RNA was subsequently utilized in a reverse transcription PCR using the primer set N-LN and the N protein as a marker (see Section 3.6 for conditions).

Results indicated a difference in the amount of viral RNA detected between the three groups which can be quantified using the qT-PCR assay.



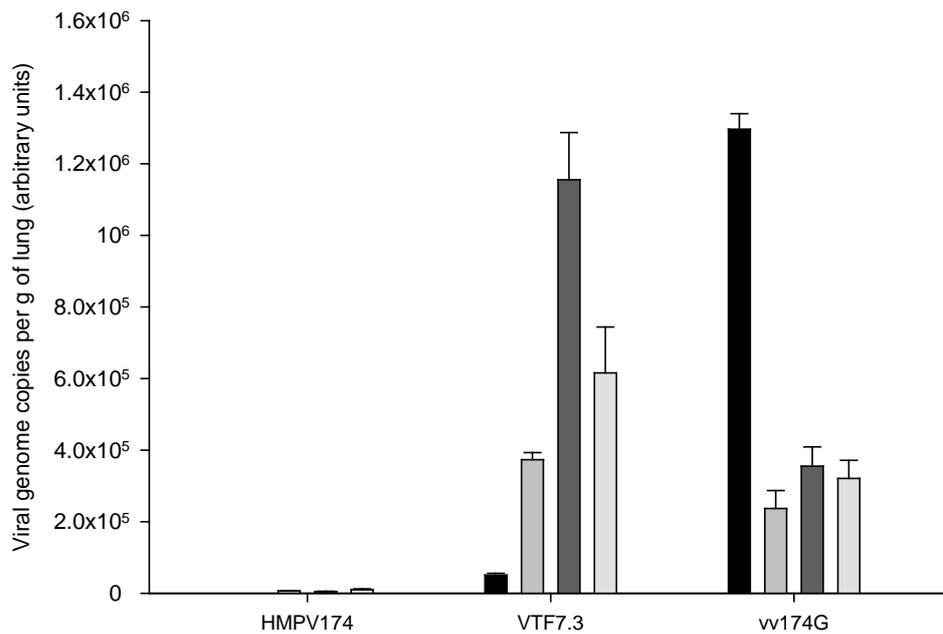
**Figure 6.16 Amplification of N protein RNA extracted from lungs lysates (a) and nasal mucosa samples (b) obtained in the challenge experiment.**

Lanes are as follows; 1 – 1Kb molecular marker, 2 to 5 – HMPV immunised mice; 6 to 9 – VTF7.3 immunised mice; 10 to 13 – vvG immunised mice; 14- PCR positive control; 15 – PCR negative control; 16 – 1Kb molecular marker.

## 6.8.6 QT-PCR

### 6.8.6.1 Lungs lysates

Quantitative PCR was performed on the RNA samples extracted, as described in 3.6.8. The results presented in Figure 6.17 show mice immunised with whole virus had a mean of  $10^{3.83}$  ( $\pm 10^{0.16}$ ) viral genome copies, whereas mice immunised with VTF7.3 and vv174G5/VTF7.3 had a mean of  $10^{5.53}$  ( $\pm 10^{0.59}$ ) and  $10^{5.64}$  ( $\pm 10^{0.33}$ ) viral genome copies respectively. A one-way ANOVA revealed there to be a significant difference between the numbers of viral genome copies recovered from the lungs of HMPV174 immunised mice and both VTF7.3 and vv174G5/VTF7.3 immunised mice ( $P=0.001$ ,  $F=19.37$ ). There was no significant difference between the numbers recovered from VTF7.3 immunised mice and vv174G5/VTF7.3 immunised mice.

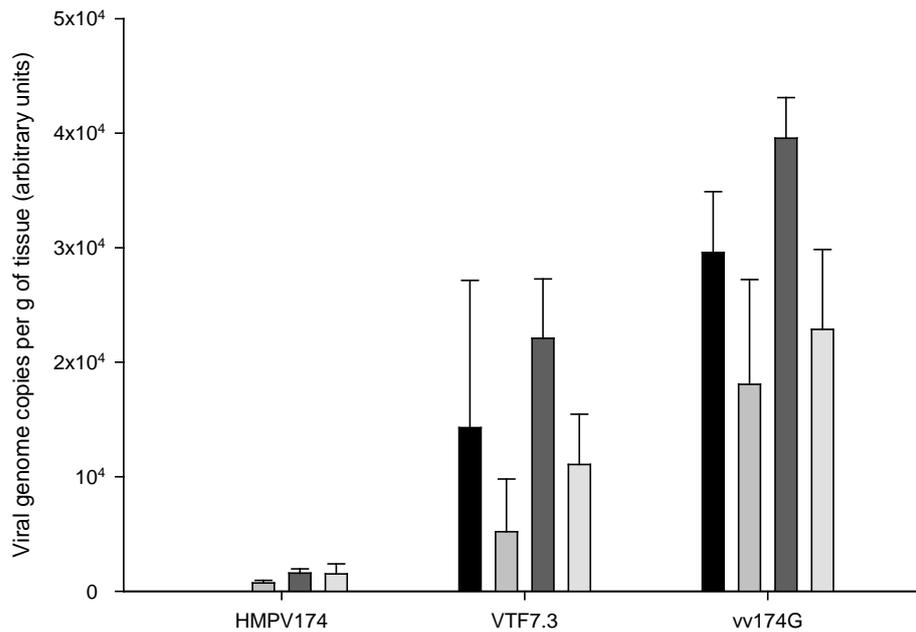


**Figure 6.17** Quantity of viral RNA present in the lungs of post-challenge mice (each bar represents one mouse)

### 6.8.6.2 Nasal mucosal samples

Samples from the nasal mucosa were treated in the same way as above and results are presented in Figure 6.18. Viral RNA recovered from the HMPV174, VTF7.3 and vv174G5/VTF7.3 immunised mice had a mean of  $10^{3.08}$  ( $\pm 10^{0.19}$ ),  $10^{4.06}$  ( $\pm 10^{0.26}$ ),  $10^{4.42}$  ( $\pm 10^{0.15}$ ) viral genome copies respectively. A one-way ANOVA revealed there to be significant difference between the numbers of viral genome copies recovered from the nasal mucosa of HMPV174 immunised mice and both VTF7.3 and vv174G5/VTF7.3 immunised mice ( $P=0.000$ ,  $F=37.09$ ). There was no significant difference between the numbers recovered from VTF7.3 immunised mice and vv174G5/VTF7.3 immunised mice.

The results showed that immunisation with whole virus but not the G glycoprotein alone protects the upper respiratory tract against HMPV challenge.



**Figure 6.18** Quantity of viral RNA present in the nasal mucosa of post-challenge mice (each bar represents one mouse)

### 6.8.7 Histochemistry and Immunohistochemistry

Histological results revealed there to be no significant difference between mice immunised with HMPV174, VTF7.3 or vv174G5/VTF7.3. Lungs had moderate levels of peri-bronchiolar and peri-vascular inflammation with a large population of lymphocytes. In certain preparations, signs of granulation were evident along with interstitial and sub-pleural inflammation but these manifestations were not associated with a particular group. These results were also observed in mice that had been immunised with vv174G5/VTF7.3 but challenged with non-infected 16HBE140 cells but not in mice who had only received vv174G5/VTF7.3 immunisations.

Immunolabelling revealed extremely low levels of viral protein expression which was only evident in certain sections and not specific for any particular group.

## Chapter 7: SH glycoprotein

The SH glycoprotein is the third membrane protein and its function is yet unknown. HMPV128 and 174 SH vaccinia virus recombinants were prepared using the approach successful for the generation of F and G glycoprotein recombinants.

### 7.1 Production of recombinant pTM1 HMPV clones

#### 7.1.1 HMPV128

##### 7.1.1.1 PCR of HMPV128 SH gene

Searching the NCBI nucleotide database for the HMPV SH gene sequence, five B subtype sequences share high levels of homology for the first 35 nucleotides. For PCR design, the first 31 nucleotides including the start codon were used for the forward primer. The homology between sequences at the 5' end was not high enough to produce a strong consensus sequence therefore the reverse primer was designed around the start of the adjacent gene, the G gene. The first 23 nucleotides after the G gene start codon were used as a primer template. The start codon is followed by an adenine allowing the restriction enzyme, *BspHI* to be used for insertion into pTM1 (see Section 4.3.2.1). The entire gene was checked for the presence of restriction enzyme sites, and *BspHI* and *XhoI* were not found allowing these sites to be integrated into the forward and reverse primers respectively.

(*BspHI*) TCATGA - HMPV SH gene - SH / G intergenic region - start of G gene - *XhoI*  
Start codon

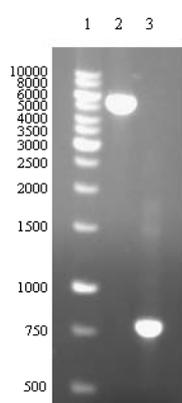
As the reverse primer is located in a separate gene, the forward primer which binds to genome sense RNA was used in the reverse transcription step as the transgenic region is required for amplification.

RNA was extracted from HMPV128, passage 10 (Section 3.5.2) and RT PCR performed using the primer set 3 (Table 3.6) under the AliT2 conditions described in

Table 3.9 and 3.12. A band of approximately 770bp was produced when the reaction mix was run on a 1% agarose gel. The product was cut from the gel and purified as described in section 3.6.7.

#### 7.1.1.2 Digestion of HMPV128 SH gene PCR products and pTM1 plasmid

To facilitate the ligation of the SH gene into the pTM1 plasmid, gel purified HMPV SH gene product was digested with restriction endonucleases *BspHI* and *XhoI*, whilst pTM1 were digested with *NcoI* and *XhoI*. Reactions were incubated for 3 hours and run on a 1% agarose gel. The results are displayed in Figure 7.1.



**Figure 7.1 Plasmid pTM1 and HMPV128 SH gene restriction digest.**

The lanes were loaded as follows; 1 - 1kb molecular marker, 2 - pTM1 digested with *NcoI* and *XhoI*, 3 - HMPV128 SH gene digested with *BspHI* and *XhoI*.

A band of 5357bp, representing the pTM1 plasmid, and a band of 770bp, corresponding to the SH gene were identified and removed with a clean scalpel. Using a QIAquick gel extraction kit, bands were purified and eluted in EB buffer (Section 3.6.7), ready for ligation.

#### 7.1.1.3 Ligation and transformation of pTM1 with HMPV128 gene products

18 different ligation reactions were carried out as described in section 3.7.6. Once ligation products were produced, they were transformed into *E. coli* TG1 (Section 3.7.7) and spread onto LB agar plates containing ampicillin. The results are shown in Table 7.1.

One colony from each successful plate was taken and grown up overnight in 5ml of LB containing ampicillin. To check the validity of the clones, plasmid DNA was prepared from 1.5ml of the overnight culture using a QIAgen miniprep spin column

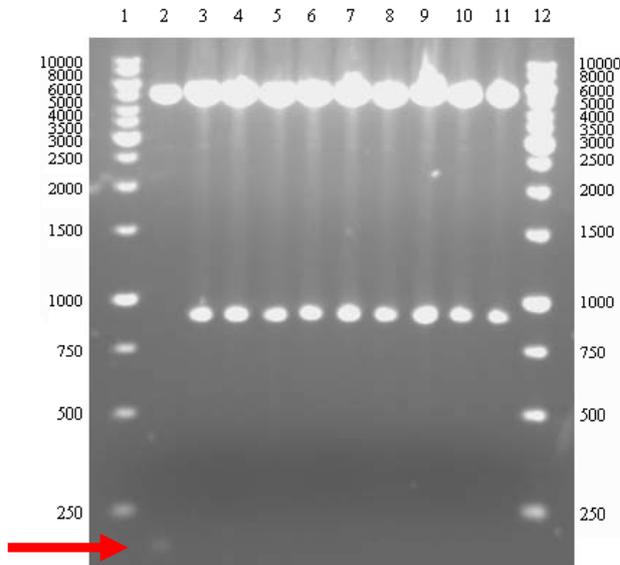
(Section 3.7.8). The DNA was eluted in 50µl of EB buffer (supplied in kit) and digested with *KpnI* and *XhoI* as in section 3.7.5. The results are presented in Figure 7.2.

**Table 7.1 Number of colonies counted after transformation of bacteria with pTM1.HMPVSH128**

<b>Reaction</b>	<b>pTM1.HMPV128SH</b>
<b>Vector only 2µl</b>	0
<b>Vector only 8µl</b>	0
<b>Vector only 20µl</b>	0
<b>Vector + insert 1:1, 2µl</b>	9
<b>Vector + insert 1:1, 8µl</b>	33
<b>Vector + insert 1:1, 20µl</b>	45
<b>Vector + insert 2:5, 2µl</b>	82
<b>Vector + insert 2:5, 8µl</b>	167
<b>Vector + insert 2:5, 20µl</b>	194
<b>Vector + insert 1:3, 2µl</b>	15
<b>Vector + insert 1:3, 8µl</b>	57
<b>Vector + insert 1:3, 20µl</b>	122
<b>Vector + insert 1:5, 2µl</b>	55
<b>Vector + insert 1:5, 8µl</b>	223
<b>Vector + insert 1:5, 20µl</b>	248
<b>No DNA control</b>	0
<b>No DNA control</b>	0
<b>No DNA control</b>	0

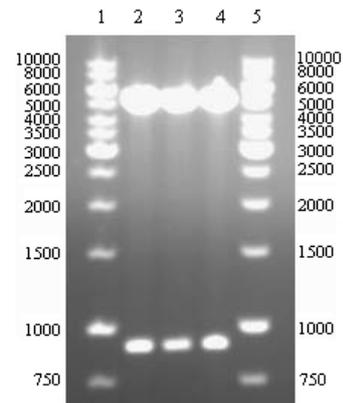
The results presented in Figure 7.2 revealed all nine transformed bacterial colonies digested into two bands, one corresponding to the pTM1 plasmid (5257bp) and another representing the SH gene (870bp – 770bp of SH gene plus 100bp of plasmid). Lane 2 represented pTM1 without an insert to define the length cut out by digesting with restriction endonucleases *KpnI* and *XhoI*. One of the transformed clones (pTM1.128SH clone 7) was chosen and streaked onto a LB agar plate containing ampicillin before being grown overnight in LB selective medium.

DNA was prepared as before and digested with both *KpnI* and *XhoI* restriction endonucleases to ensure stability of the culture (Figure 7.3).



**Figure 7.2 Restriction digest of transformation of pTM1 with the HMPV128SH gene.**

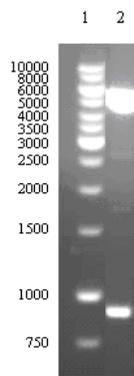
The lanes are as follows; 1 - 1Kb molecular marker, 2 – pTM1, 3 to 11 – various colonies picked, 12 – 1Kb molecular marker.



**Figure 7.3 Restriction digests of cloned recombinant pTM1 with HMPV128 SH gene.**

The lanes are as follows; 1 - 1Kb molecular marker, 2 to 4 - colonies picked from one clone, 5 – 1Kb molecular marker.

One of these clones (pTM1.128SH clone 7.1) was subsequently used for large scale production of recombinant plasmid DNA using a QIAgen maxiprep kit following the manufacturers instructions. The concentration of DNA yielded 2.0 mg/ml. The eluted DNA was digested with restriction endonucleases *KpnI* and *XhoI* and the results are presented in Figure 7.4.



**Figure 7.4 Restriction digest of pTM1.128SH8.1 maxiprep.**

Lanes are as follows; 1 - 1Kb molecular marker, 2 - pTM1.128SH8.1.

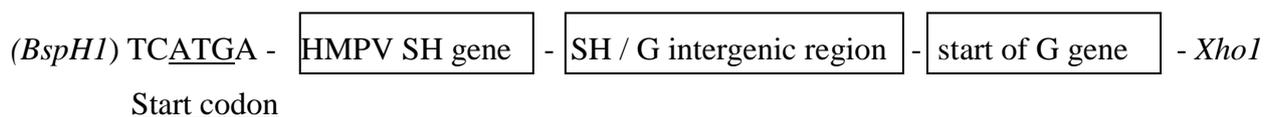
Once cloned into pTM1, the gene was sequenced in both directions from primers HMPV128SHF and HMPV128SHR (primer set 3, Table 3.6) and cross referenced against the database sequences.

Nucleotide and amino acid identities are presented in Table 7.4 and conclude that the SH glycoprotein of HMPV128 is most closely related to JPS02/76 B1. Upon evaluation of the amino acid structure, it appeared there was a stop codon caused by an amino acid substitution at residue 203 (Site B, Table 7.2). This would therefore only allow 67 amino acids to be translated with approximately 60 amino acids located in the cytoplasmic and transmembrane domain (Collins and Crowe, 2007). Despite being a truncated form of the SH gene, work was continued to allow expression in the vaccinia virus system.

## 7.1.2 HMPV174

### 7.1.2.1 PCR of HMPV174 SH gene

Searching the NCBI nucleotide database for the HMPV SH gene sequence, six A strain sequences share high levels of homology for the first 35 nucleotides. For PCR design, the first 31 nucleotides including the start codon were used for the forward primer. The homology between sequences at the 5' end was not high enough to produce a strong consensus sequence therefore the reverse primer was designed around the start of the adjacent gene, the G gene. The first 23 nucleotides after the G gene start codon were used as a primer template. The entire gene was checked for the presence of restriction enzyme sites, and *BspHI* and *XhoI* were not found allowing these sites to be integrated into the forward and reverse primers respectively.



As the reverse primer is located in a separate gene, the forward primer which binds to genome sense RNA was used in the reverse transcription step as the transgenic region is required for amplification.

RNA was extracted from HMPV174, passage 11 (Section 3.5.2) and RT PCR performed using the primer set 4 (Table 3.8) under the AliT2 conditions described in Table 3.9 and 3.12. A band of approximately 770bp was produced when the reaction mix was run on a 1% agarose gel. The product was cut from the gel and purified as described in section 3.6.7.

### 7.1.2.2 Site directed mutagenesis

HMPV174 SH product was purified as described in section 3.6.7 and digested with restriction endonucleases *BspHI* and *XhoI*, whilst pTM1 were digested with *NcoI* and *XhoI*. Reactions were incubated for 3 hours followed by visualisation on a 1% agarose gel (data not shown). Interestingly, two bands (250bp and 500bp) appeared on the gel and after sequencing the gene in both directions (using the primer set 4, Table 3.8), a *BspHI* restriction site was identified between bases 267 and 272 (Figure 7.5). As no other restriction endonuclease could be employed, site directed mutagenesis was performed. Forward (HMPV174SHF2) and reverse (HMPV174SHR2) primers were designed to overlap the restriction site and substitute a thymine for a cytosine preserving the asparagine amino acid (see Figure 7.5).

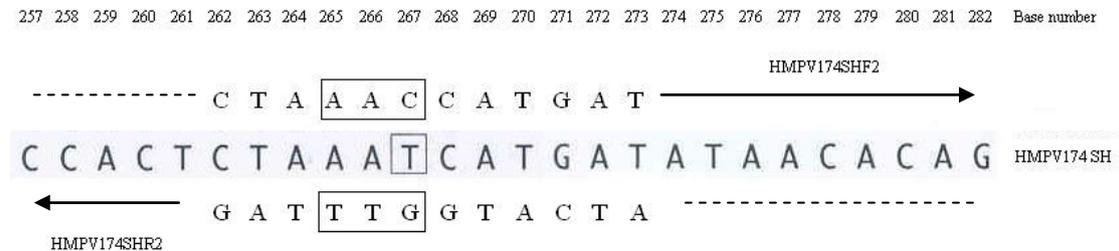
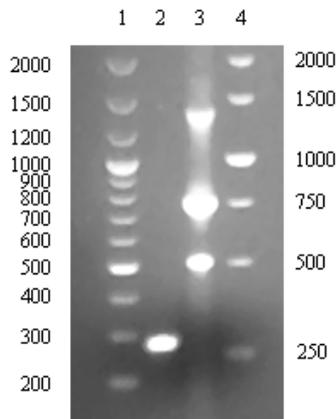


Figure 7.5 Schematic diagram of HMPV174 SH gene from bases 257 to 282.

RNA extracted from HMPV174 infected 16HBE140 cells (passage 11,  $2.6 \times 10^6$  ffu/ml, Section 3.5.2) was reverse transcribed using the HMPV174SH F primer according to the conditions stated in Table 3.9 and 3.12. Using the AliT2 cycling programme, amplification of the SH gene was then performed using the HMPV174SH F / HMPV174SH R2 and the HMPV174SH F2 / HMPV174SH R

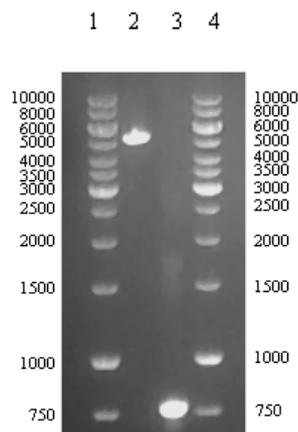
primer sets in two separate reactions to produce bands of approximately 250bp and 500bp respectively. The results are presented in Figure 7.6



**Figure 7.6 Amplification of fragments of the HMPV174 SH gene after site directed mutagenesis.**

Lanes are as follows; 1 – 100bp molecular marker; 2 – results of HMPV174SH F and HMPV174SH R2; result of HMPV174SH F2 and HMPV174SH R; 4 – 1Kb molecular marker.

The multiple bands present in lane three represent the target 500bp amplified product as well as unidentified 750bp and 1400bp bands. Bands at 250bp (lane 2) and 500bp (lane 3) were extracted and purified (Section 3.6.7) and the eluate added to a PCR mix containing the primer set 4 (Table 3.8) and run under the AliT2 conditions stated in section 3.6.5. A band of 750bp was evident when run on a 1% agarose gel (data not shown) suggesting the site directed mutagenesis had been successful. This was retrieved from the gel with a clean scalpel and DNA extracted (Section 3.6.7) before being digested with restriction endonucleases *BspHI* and *XhoI* alongside the pTM1 plasmid digested with *NcoI* and *XhoI*. The results presented in Figure 7.7 reveal the *BspHI* restriction site at position 267 has been eradicated and the SH gene is ready for ligation into the pTM1 plasmid.

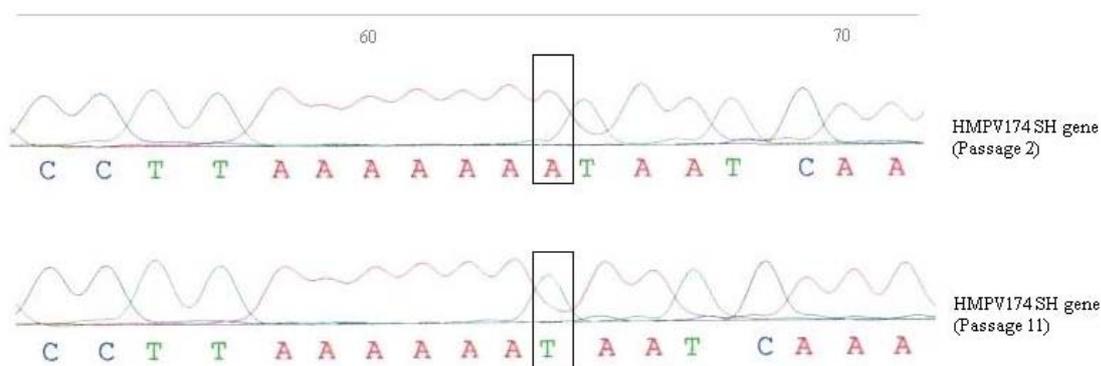


**Figure 7.7 Plasmid pTM1 and HMPV174 SH gene restriction digest.**

Lanes are as follows; 1 – 1Kb molecular marker; 2 – pTM1 plasmid digested with digested with *NcoI* and *XhoI*, 3 – HMPV174 SH gene digested with *BspHI* and *XhoI*.



Since a truncated form of the SH gene from HMPV128 was already cloned into pTM1 (Section 7.1.1), RNA from an earlier passage of HMPV174 (passage 2, Section 3.5.2) was extracted from HMPV174 infected 16HBE140 cells as described in section 3.6.2.1 and amplified using primer set 4 (Table 3.8) under the conditions described in section 3.6.5 (RT step and AliT2). The SH gene was then sequenced in both directions using the same primer set to establish whether the frame shift mutation was present. Analysis of the chromatogram (Figure 7.10) revealed there to be no nucleotide deletion at position 64 (see Appendix 3). Following the procedure outlined in section 7.1.2.2, HMPV174 SH gene from passage 2 was cloned into pTM1 to allow presentation of the wild type SH gene.



**Figure 7.10 Chromatogram section of passage 2 and 11 of HMPV174 SH gene**

### **7.2.1 Comparison of the SH gene from a variety of strains of HMPV**

Discovering both HMPV174 and HMPV128 SH genes had mutated to non-functional forms at high passage, HMPV145 (passage 9, Section 3.5.2) was sequenced for comparison. Surprisingly, results revealed there to be the same frame shift mutation at passage 11 as in HMPV174, resulting in a 20 amino acid truncated SH gene (Table 7.2). To ensure these mutations were not a characteristic of the specimens isolated from nasopharyngeal secretions, early passages (passage 2) of both HMPV174 and 145 were sequenced. There were no mutations in the early passages of either of the strains indicating the mutations must have occurred as a result of passage in cell culture. As a confirmation, two further subgroup A strains (NCL03-4/228 and

NCL03-4/230) at passage two were sequenced. Both possessed a wildtype genotype (Appendix 3). An earlier passage of HMPV128 (passage 2) could not be isolated without the mutation.

**Table 7.2 Sequence analysis of the SH gene from a variety of HMPV strains at two mutation sites**

HMPV strain	Passage	Site A (residue 64)	Site B (residue 203)	Mutation
HMPV174	2	AAAAAAATAA	CTGAGTCAGA	-
	11	AAAAAA - TAA	CTGAGTCAGA	Frame shift
HMPV145	2	AAAAAAATAA	CTGAGTCAGA	-
	9	AAAAAA - TAA	CTGAGTCAGA	Frame shift
HMPV128	10	AAAAAAATAA	CTGAGTAAAGA	Stop codon
HMPV228	2	AAAAAAATAA	CTGAGTCAGA	-
HMPV230	2	AAAAAAATAA	CTGAGTCAGA	-

Each gene was evaluated for both its nucleotide and amino acid identity with either HMPV128, HMPV145 or HMPV174 to remove the possibility of cross contamination (Table 7.3). These genes were also evaluated for both their nucleotide and amino acid identity with strains from the NCBI database representing all four lineages (Table 7.4).

**Table 7.3 Percentage of amino acid or nucleotide sequence identity between HMPV128 (passage 10), HMPV145 (passage 2) and HMPV174 (passage 2) SH genes**

HMPV genes	Percent amino acid sequence identity (percent nucleotide sequence identity <sup>†</sup> )		
	HMPV128 vs HMPV145	HMPV128 vs HMPV174	HMPV145 vs HMPV174
SH	58 (68)	59 (69)	98 (99)

<sup>†</sup> Amino acid sequence identities were calculated based on the complete predicted proteins; in the case of G, overhangs on the carboxy-terminal side of the alignments due to length differences were not included in the calculations. Nucleotide sequence identities are shown in parentheses and are based on the protein-coding sequence exclusive of non-coding sequences.

**Table 7.4 Percentage of amino acid or nucleotide sequence identity between HMPV128, 145 and 174 SH genes and other HMPV strains from the NCIB database**

HMPV genes compared	Percent amino acid sequence identity (percent nucleotide sequence identity <sup>†</sup> )			
	JPS03/180 (A1)	CAN97/83 (A2)	JPS02/76 (B1)	CAN98/75 (B2)
<b>Passage 10 HMPV128 SH</b>	59 (72)	60 (70)	94 (96)	81 (86)
<b>Passage 2 HMPV145 SH</b>	85 (90)	95 (94)	60 (69)	54 (66)
<b>Passage 2 HMPV174 SH</b>	85 (89)	96 (94)	60 (69)	55 (66)
<b>Passage 11 HMPV174SH</b>	23 (89)	23 (94)	21 (69)	22 (66)

<sup>†</sup> Amino acid sequence identities were calculated based on the complete predicted proteins; in the case of G, overhangs on the carboxy-terminal side of the alignments due to length differences were not included in the calculations. Nucleotide sequence identities are shown in parentheses and are based on the protein-coding sequence exclusive of non-coding sequences.

Analysis of the results reveal HMPV128 SH gene is most closely related to JPS02-76 (B1) and passage 2 HMPV174 SH gene to CAN97/83 (A2) corresponding to the results obtained from the analysis of the F and G genes. The marked differences observed at the amino acid level with passage 11 of HMPV174 are a result of the frame shift mutation at nucleotide residue 64. However, HMPV145 SH gene most closely resembles CAN97/83 not JPS02-76. It thus differs from HMPV145 F and G genes but corresponds to the N gene sequence analysis. The possibility of contamination from the other strains has been removed since sequence analysis confirms each gene differs at both nucleotide and amino acid level from its counterpart. Furthermore, the likelihood of contamination with another A2 strain not discussed in this thesis is impossible since the SH gene of HMPV has not been amplified before this project.

### **7.2.1.1 Ligation and transformation of pTM1 with HMPV174 gene products**

18 different ligation reactions were carried out between the amplified SH gene of HMPV174 passage 2 and pTM1 as described in section 3.7.6. Once ligation products

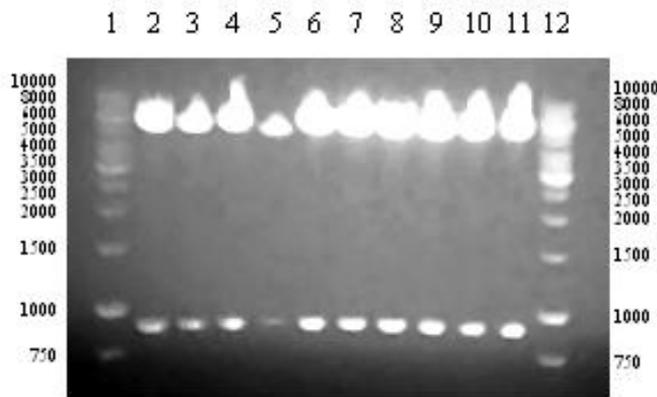
were produced, they were transformed into *E. coli* TG1 (Section 3.7.7) and spread onto LB agar plates containing ampicillin. The results are shown in Table 7.5.

**Table 7.5. Number of colonies counted after transformation of bacteria with pTM1.HMPVSH174**

<b>Reaction</b>	<b>pTM1.HMPV174SH</b>
<b>Vector only 2<math>\mu</math>l</b>	0
<b>Vector only 8<math>\mu</math>l</b>	0
<b>Vector only 20<math>\mu</math>l</b>	0
<b>Vector + insert 1:1, 2<math>\mu</math>l</b>	0
<b>Vector + insert 1:1, 8<math>\mu</math>l</b>	2
<b>Vector + insert 1:1, 20<math>\mu</math>l</b>	6
<b>Vector + insert 2:5, 2<math>\mu</math>l</b>	3
<b>Vector + insert 2:5, 8<math>\mu</math>l</b>	21
<b>Vector + insert 2:5, 20<math>\mu</math>l</b>	42
<b>Vector + insert 1:3, 2<math>\mu</math>l</b>	0
<b>Vector + insert 1:3, 8<math>\mu</math>l</b>	8
<b>Vector + insert 1:3, 20<math>\mu</math>l</b>	2
<b>Vector + insert 1:5, 2<math>\mu</math>l</b>	8
<b>Vector + insert 1:5, 8<math>\mu</math>l</b>	28
<b>Vector + insert 1:5, 20<math>\mu</math>l</b>	33
<b>No DNA control</b>	0
<b>No DNA control</b>	0
<b>No DNA control</b>	0

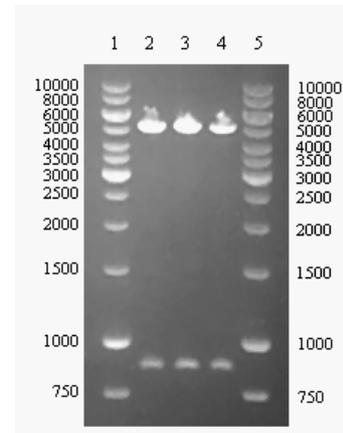
One colony from each successful plate was taken and grown up as described previously. Plasmid DNA obtained from a QIAGEN miniprep was digested with *BspHI* and *XhoI*. The results for pTM1.HMPV174SH are presented in Figure 7.11 and reveal successful transformation of all nine bacterial colonies, with each containing two bands corresponding to the pTM1 plasmid (5257bp) and the SH gene (870bp). One colony (pTM1.174SH clone 7) was chosen to be recloned and results are presented in Figure 7.12. A maxiprep pTM1.174SH clone 7.3 was performed

using a QIAGEN maxiprep kit (Section 3.7.9) and the yield of total DNA was 2.1 mg/ml.



**Figure 7.11. Restriction digest of transformation of pTM1 with the HMPV174 SH gene.**

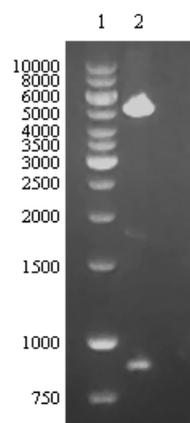
The lanes are as follows; 1 - 1Kb molecular marker, 2 to 11 – various colonies picked, 12 – 1Kb molecular marker.



**Figure 7.12. Restriction digests of cloned recombinant pTM1 with HMPV174SH gene.**

The lanes are as follows; 1 - 1Kb molecular marker, 2 to 4 - colonies picked from one clone,

To ensure the plasmid DNA was eluted correctly, pTM1.174SH7.3 DNA preparation was double digested with restriction enzymes *KpnI* and *XhoI*. The results are presented in Figure 7.13.



**Figure 7.13 Restriction digest of pTM1.174SH7.3 maxiprep.**

Lanes are as follows; 1 - 1Kb molecular marker, 2 - pTM1.174SH7.3.

### 7.3 Transient expression of HMPV.pTM1 clones in vaccinia virus

10µg/ml of DNA from pTM1.174SH7.3 and pTM1.128SH7.1 was transfected into HeLa cells along with VTF7.3 at an MOI of 30 as described in section 3.5.12. After 21 hours, the cells were tested for expression of SH antigen by immunofluorescence staining with mouse anti-HMPV174, 145 and 128 polyclonal sera (Section 3.2.1.2). The results are presented in Table 7.6.

**Table 7.6 Reactivity of mouse anti-HMPV174, 145 and 128 polyclonal serum with transiently expressed pTM1.174SH7.3 and pTM1.128SH7.3.**

Virus	Mouse polyclonal anti-HMPV antibody			MAb24
	Anti-174	Anti-145	Anti-128	
<b>pTM1.174 SH clone 7.3</b>	-/+	+	+	-
<b>pTM1.128 SH clone 7.1</b>	-/+	+	+ /+++	-
<b>VTF7.3 infected HeLa (not transfected)</b>	-/+	+	+	-
<b>HeLa cells (not infected or transfected)</b>	-/+	+	+	-

- No antibody staining
- + Small number of cells with a small amount of antibody staining
- ++ Approximately 50% of cells with specific antibody staining
- +++ Approximately 80% or more cells with specific antibody staining
- ++++ Approximately 100% or more cells with specific antibody staining

There was weak specific staining of HMPV128 SH glycoprotein with the homologous polyclonal anti serum, demonstrating that the truncated form of the SH glycoprotein is very poorly immunogenic but can still elicit an immune response. It is not however cross-reactive with the subgroup A strain. However, there was no specific staining of polyclonal anti – HMPV174 or 145 antibodies with either of the transiently expressed SH glycoproteins. It is likely that the immunogen used to generate the anti-HMPV174 and 145 polyclonal mouse sera carried the SH gene mutation and as a result would not have generated antibody directed towards the luminal domain. It cannot be determined from this experiment whether the truncated SH protein in HMPV174

passage 11 was non-immunogenic or that protein is not being expressed from pTM1.174SH clone 7.3.

#### 7.4 Production of vaccinia virus recombinants

As there is no way to detect the expression of the functional SH gene in the HMPV174 recombinant, only vaccinia virus recombinants were prepared from the HMPV128 SH gene. Vaccinia virus clones were produced by initially infecting CV-1 cells with wild type vaccinia virus at an MOI of 0.05 subsequently transfected with 3µg/ml of DNA from pTM1.128SH7.1 maxiprep (Section 3.5.13). Transfected infected CV-1 cell lysates were then inoculated onto TK-143 cells under BuDR selection pressure, overlaid with agarose and neutral red to allow virus plaques to become visible (Section 3.5.14). Several individual plaques were collected and subsequently cultured in a 24 well plate of TK-143 cells to produce a stock for DNA preparation (Section 3.5.15).

##### 7.4.1 PCR of DNA preps from recombinant vaccinia virus

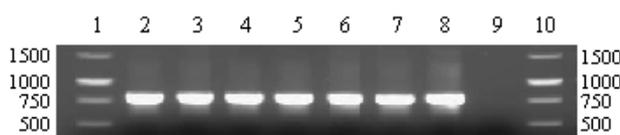
DNA was purified from vv128SH recombinant vaccinia virus clones 1 to 6 (Section 3.5.16). The DNA was amplified by PCR using primer set 3 (Table 3.6) under the AliT2 reaction conditions as described in Table 3.9 and 3.12. The amplicand was resolved on a 1% agarose gel and visualised with ethidium bromide using ultraviolet light. The results are presented in Figure 7.14.



**Figure 7.14 PCR products of HMPV128 SH gene DNA preps.**

Lanes are as follows; 1 – 1Kb molecular marker, 2 to 5 – several SH clones, 6 – pTM1.128SH maxiprep at dilution  $10^{-3}$ ; 7 - negative PCR control, 8 – 1Kb molecular marker

Only one out of five potential vaccinia virus recombinants produced a band at just over 750bp corresponding to the HMPV128 SH gene (Figure 7.14). Plaques 2 was recloned on TK-143 cells in the presence of BuDR and all plaques picked contained the gene of interest (Figure 7.15).



**Figure 7.15. PCR products of recloned SH gene DNA preps of HMPV128 SH clone 3**

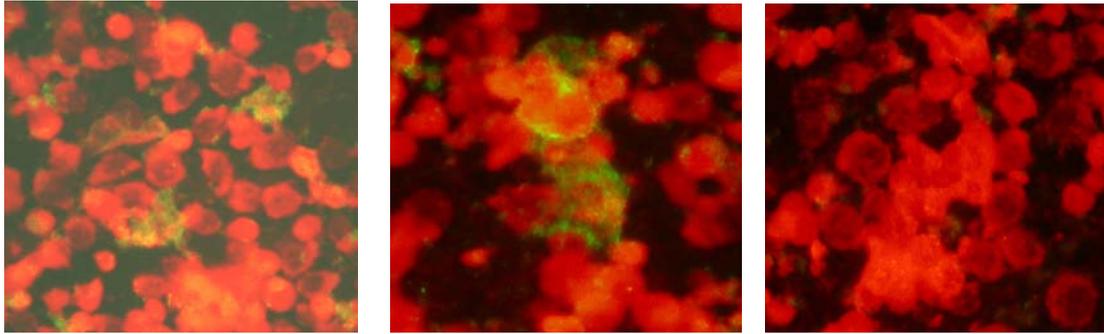
Lanes are as follows; 1 – 1Kb molecular marker, 2 to 7 – several SH clones, 8 – pTM1.128SH maxiprep at dilution  $10^{-3}$  positive control, 9 – negative PCR control, 10 – 1Kb molecular marker

## 7.5 Co-expression of HMPV vaccinia recombinants

The recombinant vaccinia virus clone vv128SH2.1 was grown up to high titres by successive passage in L cells as described in section 3.5.15. Once passaged into  $75\text{cm}^3$  tissue culture flasks, the clone was titred according the method described in section 3.5.11.

The recombinant vaccinia virus clone was coinfecting into L cells with VTF7.3 at an MOI of 3 as described in section 3.5.17. They were incubated for 24 hours. Spot slides were produced and the remainder of the preparation was inactivated with BEI (Section 3.5.18) and further spot slides prepared.

The slides were tested for expression of the HMPV128 SH antigen by immunofluorescence staining with both MAb24 and mouse polyclonal anti-HMPV128 serum (Figure 7.16).



**Figure 7.16** Example of immunofluorescence staining of L cells (a) coinfecting with vv128SH2.1 and VTF7.3 (b) coinfecting with vv128SH2.1 and VTF7.3 after inactivation and (c) VTF7.3 only (before inactivation), all stained with anti-HMPV128 polyclonal antibody.

Polyclonal anti-HMPV128 mouse serum produced specific staining of the coinfecting L cells both before and after inactivation, similar to that of the transiently expressed protein (Table 7.6). There was no staining with MAb24 confirming the SH glycoprotein is expressed alone.

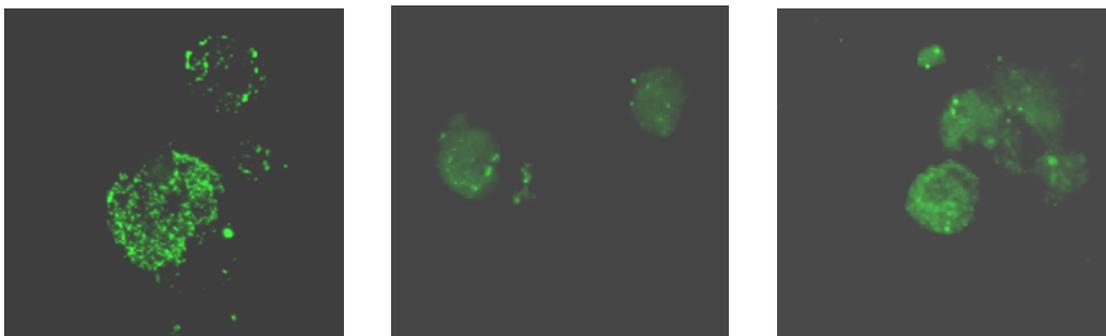
### 7.6 Preparation of mono-specific mouse anti-sera

Polyclonal monospecific antibody was prepared as before (Section 4.9) by immunising mice with inactivated vv128SH2.1/VTF7.3 antigen. Forty-nine days after the initial subcutaneous inoculation, the serum antibody titres (Table 7.7) were established by titrating the final cardiac puncture by indirect immunofluorescence staining on both homologous and heterologous HMPV grown in 16HBE140 cells (see Section 3.5.1).

**Table 7.7** Antibody titres of monospecific anti-HMPV128 SH mouse serum on HMPV infected 16HBE140 cell cultures.

Virus	Anti-128 SH (Titre log <sub>10</sub> )
HMPV174	3.11
HMPV145	3.11
HMPV128	3.11

To investigate whether the antibody preparation was targeting the cytoplasmic domain of the glycoprotein, the serum sample was tested using membrane immunofluorescence (Section 3.5.5). Live HMPV infected 16HBE140 cells were harvested and incubated with the monospecific antibody for 1 hour before being gently washed and stained with SAM FITC for detection by fluorescence microscopy. No membrane specific staining with the anti – HMPV128 SH antibody on either the HMPV128 infected cells or the uninfected 16HBE140 cells was observed. This suggests either the antibody is targeting either the cytoplasmic region or the transmembrane region of the protein as specific immunofluorescence can be observed in the fixed cell preparation or the truncated SH protein is not transported to the cell surface.



**Figure 7.17** Example of membrane immunofluorescence staining in (a) HMPV128 infected cells stained with monospecific anti-vv128F5.5/VTF7.3 antibodies, (b) HMPV128 infected cells and (c) uninfected 16HBE140 cells stained with monospecific anti-vv128SH2.1 antibodies.

### **7.6.1 Neutralisation**

HMPV174 (passage 2) and HMPV128 (passage 10) infected 16HBE140 cell preparations were incubated with vv128SH2.1/VTF7.3 monospecific antibody to produce a final dilution series of 1/10 to 1/2560 and inoculated onto 16HBE140 cells as described in section 3.5.8. At 15 days post-infection, spots were made and tested by immunofluorescence using HMPV monoclonal antibody pool (Section 3.5.4).

Each spot was rated by the number of infected cells together with the intensity of fluorescence in comparison with the positive control as described in Table 7.6. The

cut off for neutralisation was < ++ (where the positive control was +++) and 50% neutralisation titres were calculated according to the Reed and Muench method. The results are displayed in Table 7.8.

**Table 7.8 Neutralisation of HMPV128 with all monospecific antibodies generated**

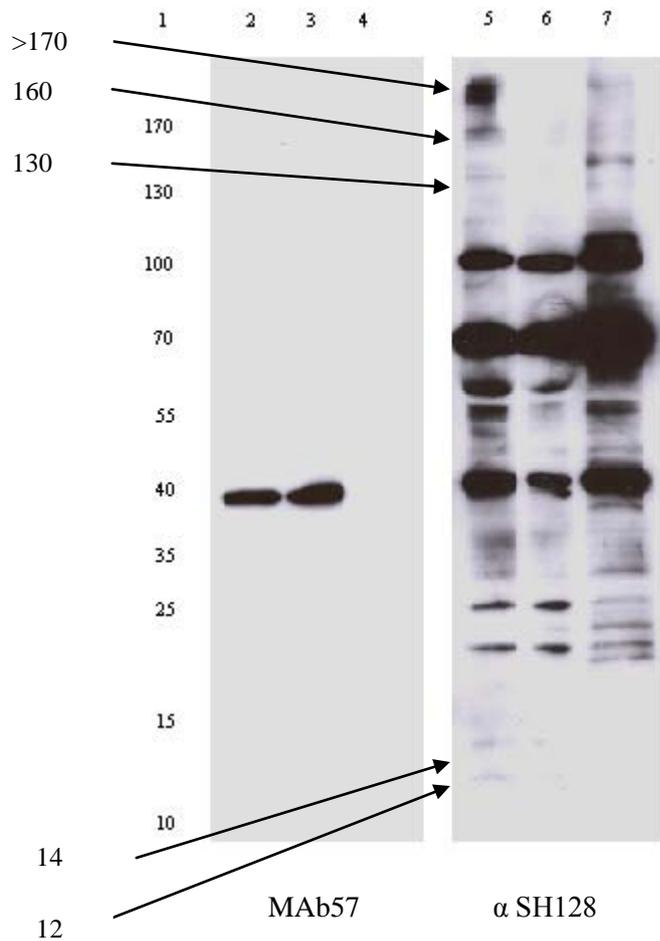
Monospecific antibody	50% serum neutralising antibody titre	
	(log <sub>10</sub> )	
	HMPV174 (P2)	HMPV128 (P10)
<b>α vv128SH2.1/VTF7.3</b>	3.25	1.9
<b>α VTF7.3</b>	1.75	1.75
<b>Normal mouse serum</b>	0	0

Whilst anti-VTF7.3 antibodies have a low level of neutralising activity, anti-SH128 antibodies neutralise HMPV174 to a much higher titre ( $10^{3.25}$ ) suggesting an added neutralisation effect by anti-SH antibodies. However, the neutralisation effect of these antibodies on HMPV128 is similar to that seen with anti-VTF7.3 antibodies.

### 7.6.2 Western blot

Uninfected and infected passage 2 HMPV174 and passage 10 HMPV128 cell cultures were prepared as before (Section 4.10.3) separated by polyacrylamide gel electrophoresis alongside molecular weight markers as detailed in section 3.20 and blotted onto the PVDF membrane (Section 3.21). The membrane was stained with MAb57 at a concentration of 5µg/ml and monospecific anti vv128SH2.1/VTF7.3 antibody at a 1/500 dilution. The bands were visualised as before (Section 4.10.3). The monospecific anti vv128SH2.1/VTF7.3 antibody stained a number of bands in the uninfected 16HBE140 cells. However, several bands of high molecular weight were stained in the HMPV174 infected cell lysate only, one above 170KDa, one at approximately 160KDa and the other at 130KDa all potentially representing the glycosylated form of the SH glycoprotein. The antibody also stained 14KDa and 12KDa bands in both HMPV174 and HMPV128 infected cell lysates, potentially

representative of a partially digested form of the protein. All other bands appeared cross reactive with both HMPV lanes and 16HBE140 negative cells.



**Figure 7.18. Comparison of HMPV174, 128 infected and uninfected 16HBE140 cell lysate stained with either MAb57 or monospecific anti-vv128SH2.1/VTF7.3 antibody western blot.**

Lanes were as follows; 1 – molecular weight marker; 2, 5 – HMPV174 infected cell lysate; 3, 6 – HMPV128 infected cell lysate; 4, 7 – uninfected 16HBE140 cells.

Text below the figure indicates antibody used to stain each blot.

\* Apparent HMPV174 specific bands in lane 5 at 12 and 14KDa are observed in lane 6 but are not visible in figure.

### 7.7 Sequencing virus recovered from infected mice

Infectivity results from mice infected with passage 11 / 12 HMPV174 (Chapter 6) were surprising since RNA could be easily isolated by quantitative PCR from all samples taken yet no infectious virus could be cultured *in vitro*. With the discovery of all high passage HMPV strains having a non functional SH gene, both the inoculum and RNA extracted from the lungs of all mouse studies were sequenced in both directions using primer set 4 (Table 3.8). The results presented in Table 7.9 revealed all inocula used in the animal studies possessed the non-functional SH gene apart from that in the original growth curve conducted with lower passage virus (Passage 4) which contained a mixed population of both wild type and mutated virus. Of two samples of infected lung from this experiment, collected on day 5 post-infection, one

yielded a mixed population of virus genome whilst the second yielded only wild type genome. Virus genome recovered from the lungs of all mice inoculated with mutant virus yielded only mutant genomes.

**Table 7.9 Sequence analysis of the HMPV174 SH gene from inoculum used in the mouse model experiments**

Inocula	Passage	Site A (residue 64)	Mutation
<b>5 x 10<sup>5</sup> ffu/ml</b>	4	TAAAAAAA <del>A</del> TAAT	Mixed population
		TAAAAAAA - TAAT	Wild type / Frame shift
<b>2.6 x 10<sup>6</sup> ffu/ml</b>	11	TAAAAAAA - TAAT	Frame shift
<b>7 x 10<sup>6</sup> ffu/ml</b>	12	TAAAAAAA - TAAT	Frame shift
<b>Challenge</b>	11	TAAAAAAA - TAAT	Frame shift

**Table 7.10 Sequence analysis of the HMPV174 SH gene from lung homogenates extracted from the mouse model experiments**

Inocula	Passage	Samples sequenced	Site A (residue 64)	Mutation
<b>5 x 10<sup>5</sup> ffu/ml</b>	4	2	TAAAAAAA <del>A</del> TAAT	Wild type
			TAAAAAAA <del>A</del> TAAT	Mixed population
			TAAAAAAA - TAAT	Wild type / Frame shift
<b>2.6 x 10<sup>6</sup> ffu/ml</b>	11	1	TAAAAAAA - TAAT	Frame shift
<b>7 x 10<sup>6</sup> ffu/ml</b>	12	1	TAAAAAAA - TAAT	Frame shift
<b>Challenge</b>	11	1	TAAAAAAA - TAAT	Frame shift

Thus the recovery of infectious virus from the lungs of mice coincided with the recovery of wild type virus with a full SH gene. Further experimentation will be required to confirm the effect of the SH gene mutations on virus infectivity *in vivo*.

## Chapter 8: Discussion

The aims of this project were initially to develop vaccinia virus recombinants expressing the individual glycoproteins of at least two strains of HMPV, one from each sub-group. These would be used to generate both glycoprotein specific polyclonal and monoclonal antibodies and evaluate the protective effects of antibody to the individual glycoproteins in an animal model system.

### 8.1 Strains of HMPV

Two strains of HMPV were chosen for this study, HMPV174, representing the A2 subgroup and HMPV128, representing the B1 sub-group. HMPV145, a second reported A2 strain, was also chosen to investigate intra-subgroup reactivity of the antibodies generated. These viruses were isolated in Newcastle upon Tyne during the 2003 / 2004 epidemic and passaged in 16HBE140 cells to create working stocks. Sequence analysis of both the F and G genes with strains from the NCBI database revealed similar levels of variability to those observed by other groups (Bastien *et al.*, 2004; Biacchesi *et al.*, 2003; Biacchesi *et al.*, 2004b; Ishiguro *et al.*, 2004; Ludewick *et al.*, 2005; van den Hoogen *et al.*, 2002; van den Hoogen *et al.*, 2004). Whilst the F genes of both HMPV128 and 174 displayed high levels of conservation with members of homologous sub-groups from the database, resulting in a mean of 95% nucleotide identity, compared to 94% reported by others (Biacchesi *et al.*, 2003; Ludewick *et al.*, 2005). There was 83% identity between sub-groups (compared to 83-85% in similar studies (Biacchesi *et al.*, 2003; Ludewick *et al.*, 2005)). These results were mirrored at the amino acid level.

The levels of divergence observed between the G genes of both strains of HMPV also reflect those reported previously. Whilst here, a mean of 86% nucleotide identity was observed between strains belonging to the same sub-group (70-100% in similar studies), a higher degree of variation of 60% was observed between sub-groups (46-59% (Biacchesi *et al.*, 2003; Ludewick *et al.*, 2005)). These results indicate these genes are a good representation of the appropriate lineage.

Interestingly, sequence analysis of the F and G genes from HMPV145, previously reported to cluster with sub-group A2 (Ingram, 2006), aligned this strain within the B1 lineage. In contrast, the N and SH genes most closely resembled strains of the A2 lineage suggesting HMPV145 is a complex recombinant of two strains belonging to both A and B sub-groups. The possibility of contamination was eliminated since sequence analysis of all four genes confirmed degrees of variation between HMPV128, 145 and 174. As RNA recombination is a rare event, an alternative hypothesis is the presence of a mixed population in the culture. Genes from either the A2 or B1 strain were being amplified dependent on their homology with the primers. However, the results do not support this notion as both N and F gene primer sets were designed to accommodate all four HMPV lineages and would thus be expected to amplify both sequences in a mixed infection.

## **8.2 Production of recombinant vaccinia viruses**

The production of recombinant vectors allowing eukaryotic expression of the glycoproteins of HMPV has previously been reported in a number of studies. By means of a reverse genetics system, several human parainfluenza virus recombinants have been generated expressing the F, G and SH glycoproteins of HMPV (Skiadopoulos *et al.*, 2006; Skiadopoulos *et al.*, 2004; Tang *et al.*, 2005; Tang *et al.*, 2003). Skiadopoulos *et al* modified the full-length anti-genomic PIV1 cDNA (Newman *et al.*, 2002) by insertion of the HMPV F gene flanked by PIV1 gene start and gene end transcription signals upstream of the PIV1 N ORF (Skiadopoulos *et al.*, 2004). Subsequently this was repeated for the HMPV G and SH proteins which were separately inserted immediately downstream of the PIV1 P protein ORF and modified to contain the flanking PIV1 cis-acting signals (Skiadopoulos *et al.*, 2006).

Tang *et al* (2003) fashioned chimeric PIV3 containing the HMPV F gene using the chimeric bovine / human PIV3 harbouring the F and HN genes of PIV3 (Haller *et al.*, 2001; Haller *et al.*, 2000). The HMPV F gene was inserted into the PIV3 genome as a transcriptional cassette.

These previous studies were, however, designed for the evaluation of potential multivalent vaccines not as an expression system. PIV is relatively fastidious and yields of the target protein are likely to be low. In the vaccinia virus expression system, which had already been established in this laboratory and was therefore chosen for the expression of HMPV glycoproteins, the target gene is located downstream of a T7 promoter and is transcribed by the highly efficient bacteriophage T7 polymerase (Mohamed and Niles, 2004; Moss, 1991). It is one of the highest yielding eukaryotic expression systems and this together with the ease of manipulation and production of recombinant vaccinia viruses makes this system very attractive (Fuerst *et al.*, 1987). Furthermore, for the production of monospecific anti-sera and monoclonal antibodies, the ability of the virus to infect an extensive host range is extremely useful in avoiding overwhelming background anti-cell antibody response.

Using the shuttle vector pTM1 (Moss *et al.*, 1990), constructs expressing either the F or G genes of HMPV downstream of the T7 promoter were constructed allowing the initial evaluation of potential levels of protein expression via the transient expression system (Fuerst *et al.*, 1986). Detection by indirect immunofluorescence with polyclonal mouse HMPV anti-sera revealed a good level of expression with the F and G glycoproteins of all three strains. However, expression of foreign proteins using this method does have its limitations and is not adequate for monoclonal and monospecific antibody production since high concentrations of pTM1 plasmid DNA is required for each infection along with high titres of the recombinant vaccinia virus, VTF7.3, which supplies the T7 polymerase. Therefore, the production of a recombinant virus was the next step, where the target gene was inserted into the wild type vaccinia virus genome downstream of a T7 promoter. This allowed high levels of protein expression to be obtained by co-infecting with VTF7.3 (Fuerst *et al.*, 1987). F and G protein expression of both HMPV174 and 128 from recombinant viruses was detected by immunofluorescence using polyclonal HMPV anti-sera indicating that the transient expression system was a good indication of subsequent glycoprotein recombinant vaccinia virus expression.

### **8.3 Inactivation of recombinant vaccinia virus**

Inactivation of the recombinant vaccinia viruses was a necessary step to allow safe handling when utilising these reagents. The revelation that both  $\beta$ -propiolactone and formaldehyde led to an apparent alteration and destruction of epitopes upon inactivation (Hulskotte *et al.*, 1997; Robinson, 2007), led to the application of another method that was thought to preserve epitopes. Inactivation with BEI is thought to inactivate viral infectivity by reacting with nucleic acids without interaction with proteins suggesting preservation of antigenicity (Bahnemann, 1976; Bahnemann, 1990; Hulskotte *et al.*, 1997). Results after coinfection suggested that whilst the majority of epitopes as detected by the polyclonal mouse serum, were accurately conserved, some had been destroyed as indicated by the lack of detection of vv174F7/VTF7.3 with anti-F MAb24. As a consequence, the monospecific anti-sera generated, after inactivating the recombinant vaccinia viruses, may lack some specificity for BEI labile epitopes.

### **8.4 Reactivity of polyclonal anti-HMPV sera**

Production of polyclonal HMPV anti-sera allowed the detection of the individual HMPV glycoproteins expressed by vaccinia virus. The immunisation schedule used to generate these included a preliminary intranasal inoculation, thought to increase antibody titres as this results in infection (Beeler and van Wyke Coelingh, 1989), followed by two subcutaneous inoculations before antibody titres were measured. Analogous to previous reports, the antibodies generated were cross reactive to homologous strains and heterologous strains albeit to lower levels (MacPhail *et al.*, 2004; Skiadopoulos *et al.*, 2004; van den Hoogen *et al.*, 2007; Wyde *et al.*, 2005).

Whilst all HMPV anti-sera stained the vaccinia virus expressed HMPV F protein from all three strains, only the homologous sera stained the HMPV G glycoprotein confirming its high variability and diversification as previously observed by others (Biacchesi *et al.*, 2005a; Biacchesi *et al.*, 2004b; Endo *et al.*, 2008; Mok *et al.*, 2008; Skiadopoulos *et al.*, 2006). Despite the 68% amino acid identity between the G genes of the genetically related HMPV128 and HMPV145, anti-HMPV145 serum did not

react with recombinant vaccinia virus expressing HMPV128 G glycoprotein and vice versa.

However, differences in the ability of the two sub-groups to induce high levels of antibodies meant mice immunised with sub-group B strain HMPV128 received an additional subcutaneous inoculation to induce a response comparable to that to sub-group A strain HMPV174. Similar reports have suggested a difference in replication of group A and B HMPV strains resulting in the induction of lower levels of neutralising antibodies from infection with a sub-group B strain (MacPhail *et al.*, 2004; Skiadopoulos *et al.*, 2004; van den Hoogen *et al.*, 2007; Wyde *et al.*, 2005) Furthermore, a report by Vicente *et al* (2006) suggested a possibility that HMPV A strains cause more severe disease in infants possibly as a consequence of increased replication efficiency, yet this still remains controversial. As the primary immunisation here was intranasal, differences in the ability of the virus to replicate in the mouse respiratory tract might have influenced the subsequent immune response.

## **8.5 Reactivity of monospecific anti-glycoprotein sera**

The F and G glycoproteins expressed in vaccinia virus were highly immunogenic. The monospecific anti-F antibodies possessed similar characteristics to the polyclonal HMPV anti-sera in that they were cross reactive to both homologous and heterologous strains of HMPV. However, surprisingly, the anti-G antibodies were also to some extent also cross reactive. Antibodies generated to HMPV128 G protein were the most reactive with both HMPV174 and HMPV145, whereas the specific staining pattern seen with the anti-G174 antibodies was just above the background level seen with the negative controls. This result is surprising given the extensive level of sequence variability between the G proteins of the two sub-groups (Bastien *et al.*, 2004; Biacchesi *et al.*, 2003; Galiano *et al.*, 2006; Ishiguro *et al.*, 2004; Ludewick *et al.*, 2005; Peret *et al.*, 2004; van den Hoogen *et al.*, 2004). It is also inconsistent with the failure of polyclonal anti-sera raised against the whole virus to exhibit any cross reactivity for heterologous G glycoproteins expressed in vaccinia virus. The removal of the other viral proteins, which possibly contain immunodominant epitopes, may allow the generation of antibodies directed towards normally immunologically silent

regions. Although the amino acid identity between HMPV128 G and HMPV174 G proteins is 32% and there are no conserved sequences of any length, the possibility still remains that a particular antibody could be directed towards a cross reactive conformational epitope.

Poor responses to vv174G5/VTF7.3 and vv128G3.5/VTF7.3 necessitated an additional immunisation compared to vaccinia virus F preparations. Even so both anti-F antibody titres were higher. Since all preparations contained the equivalent levels of antigen, these results suggest that the F glycoprotein is more antigenic than the G glycoprotein.

## **8.6 Neutralisations**

A further aim of this study was to investigate the ability of the monospecific specific anti-sera generated to neutralise HMPV strains from both sub-groups.

Neutralisation of viruses has been defined as the ability of antibodies to abrogate virus infectivity by direct binding to the virion. The ability of antibody to bind and inactivate virus infectivity has been proposed to be mediated by a variety of different methods including: aggregation of virions, inhibition of virion attachment, inhibition of fusion with the target cell and post entry neutralisation where antibody complexed with the virion blocks further stages of replication. These mechanisms rely upon the theory that antibodies bind to critical sites on the virion surface (Dimmock, 1993).

Another theory proposed is the simple occupancy model (Parren and Burton, 2001). It is suggested that neutralisation ensues when a significant proportion of available sites on the virion surface are antibody occupied since each virion requires a certain number of unimpeded surface antigens to infect. These antibodies coat the surface of the pathogen and, only once this coating has reached critical density, inhibit virus attachment or fusion with the target cell (Klasse and Sattentau, 2002; Parren and Burton, 2001). Various factors are thought to influence antibody occupancy including the size of the virion, the number of antigens on the virion surface, the number of

antibody binding sites and the proximity of the viral antigens on the virion surface (Klasse and Sattentau, 2002).

Whatever, the neutralisation method, the presence of complement is thought to enhance neutralisation either by interfering with the virus and target cell interaction or via complement-mediated cytotoxicity (Parren and Burton, 2001).

The neutralisation results with monospecific anti-sera for HMPV glycoproteins confirmed the staining patterns observed with the monospecific antibodies by immunofluorescence. Both preparations of anti-F antibodies neutralised in the presence of complement both HMPV174 and HMPV128 up to a titre of  $10^{3.25}$ , which was not unexpected by either theory of neutralisation given that F is abundant on the surface of the virion and its apparent role in mediating viral and cell membrane fusion during infection (Hernandez *et al.*, 1996). Other groups have reported similar findings with both polyclonal (Herfst *et al.*, 2007; Skiadopoulos *et al.*, 2006; Skiadopoulos *et al.*, 2004) and monoclonal antibodies generated against the F glycoprotein (Hamelin *et al.*, 2008; Ma *et al.*, 2005; Ulbrandt *et al.*, 2008; Ulbrandt *et al.*, 2006; Williams *et al.*, 2007).

Intriguingly, the anti-G174 monospecific antibodies also neutralised homologous HMPV174 but not HMPV128. This was unexpected since it has been reported that the immunisation of recombinant PIV1 expressing the G glycoprotein of HMPV does not induce detectable neutralising antibodies (Skiadopoulos *et al.*, 2006). In addition, Biacchesi *et al.* (2004b, 2005a) reported deletion mutants lacking the G protein were highly attenuated yet capable of replication in both the upper and lower respiratory tract of hamsters and African green monkeys. It was therefore suggested that it is an accessory protein, not required for efficient assembly or release of progeny virus and not essential for virus replication. However, the proteins high level of variation, thought to be driven by host immunity suggests that it is either under greater pressure than the F protein to evolve or its structure is more forgiving of sequence variation leading to immune escape (Bastien *et al.*, 2004; Biacchesi *et al.*, 2003; Ludewick *et al.*, 2005; van den Hoogen *et al.*, 2004).

Even more surprising, anti-G128 antibodies neutralised both HMPV128 and HMPV174. This does support the results achieved in the immunofluorescence studies but there has been no previous report of cross reactive anti-G protein antibodies neutralising or non-neutralising (Bastien *et al.*, 2004; Endo *et al.*, 2008). Studies with HRSV revealed that whilst monoclonal antibodies exhibited little neutralising activity, polyclonal antiserum raised against purified G protein or several G-specific MAbS exhibited enhanced neutralisation (Johnson *et al.*, 1987b; Martinez and Melero, 1998).

A possible explanation for such results could be attributable to the phenotypic differences observed between the strains of HMPV. Unlike viruses in other studies where infection of vero / LLC-MK2 cells requires the presence of exogenous trypsin, the strains used in this study may require the G glycoprotein for attachment and entry into the host cell. Blocking of this protein by the polyclonal antibodies would therefore neutralise the virus and inhibit subsequent infection and spread. An alternative explanation may be that the G glycoprotein is not required for infection in cell culture. However, its presence on the virion surface could permit antibody of sufficient avidity to coat the virion which would sterically inhibit the attachment of the virus mediated by other virion surface components.

The differences observed in the capacity of anti-G128 antibodies to neutralise both HMPV128 and HMPV174 compared with anti-G174 antibodies which were strain specific have been mirrored in RSV studies. Evidence of this asymmetric cross reactivity / neutralisation pattern was obtained when immunisation of mice with a sub-group A virus produced cross-reactive anti-G antibodies, whilst immunisation with a sub-group B virus only generated sub-group specific antibodies (Johnson *et al.*, 1987a; Stott *et al.*, 1987).

## **8.7 Western blots**

The monospecific antibodies were further characterised by western blotting. Each polyclonal antibody was tested against both HMPV174 and HMPV128 as well as a 16HBE140 control. MAb57 was used as a positive control, staining a single HMPV specific band at approximately 40KDa. Previous work in this laboratory used MALDI-TOF analysis to demonstrate that this antibody is specific for the N protein

(Ingram, 2006). All of the monospecific antibodies gave some background staining of the blots but non-specific staining with the anti-G174 was so heavy as to render the blot uninterpretable. Antibodies to HMPV174 F, HMPV128 F and G were highly reactive with uninfected cells blotting two very heavy bands at 70KDa and 41KDa, and numerous heavier and lighter bands. This anti-cell reactivity was not apparent when the antibodies were tested by immunofluorescence staining. All antibodies also blotted a major band at approximately 62KDa in the HMPV positive lanes but there was no differentiation between anti - F and anti - G staining of either HMPV antigen. A number of hypotheses could be made to explain this 62KDa band. As monospecific anti-sera were generated by immunisation of mice with recombinant vaccinia virus grown in mouse L cells, L cells or vaccinia virus may have a shared epitope with HMPV that would allow antibodies to cross react. Alternatively the immunised mice are making a response to a 62KDa stress protein which shares an epitope with an equivalent protein in virus infected 16HBE140 cells. Finally, the HMPV F and G glycoproteins may share an epitope and the 62KDa protein represents the HMPV F protein. This is least likely as others have not reported a shared epitope in other systems.

The failure of these monospecific antibodies to detect any HMPV specific viral proteins could be attributable to the high levels of non-specific binding. It has been reported that the F precursor protein, F<sub>0</sub>, has blotted at 59KDa (Biacchesi *et al.*, 2006; Cseke *et al.*, 2007) and therefore the heavy cross reactive bands located at approximately 62KDa could be masking this observation. Furthermore, the heavy non-specific bands at approximately 100KDa in the anti-G128 blots could be masking the specific blotting of the G glycoprotein since it has been observed in PAGE at approximately 90KDa (Biacchesi *et al.*, 2004b; Biacchesi *et al.*, 2005b).

## 8.8 Hybridomas

A further aim of this study was to generate anti-G glycoprotein monoclonal antibodies preliminary directed towards HMPV174. There are currently few monoclonal antibodies available for HMPV especially directed towards the G glycoprotein. Ulbrandt *et al* (2006) isolated a panel of monoclonal antibodies to HMPV F protein

that have been found to neutralise both *in vitro* and *in vivo*. These antibodies were generated by intranasal inoculation with wild type HMPV followed by immunisations with either recombinant adenovirus or recombinant bovine parainfluenza virus expressing the HMPV F protein. One particular antibody MAb338 was tested for prophylactic efficiency in mice (Hamelin *et al.*, 2008). A similar study revealed the generation of anti-HMPV F protein monoclonal antibodies by subcutaneous immunisations with HMPV infected cells (Ma *et al.*, 2005), similar to the process used here, although cloned hybridomas were subsequently injected intraperitoneally back into BALB/c mice to facilitate collection of asites fluid.

High levels of post translational glycosylation of the G glycoprotein including mannose N –linked sugars and O – linked sugars leads to the formation of the mature protein which is expressed on the surface of the cell membrane (Liu *et al.*, 2007). Expression of the G glycoprotein in eukaryotic cells from a vaccinia virus vector allows transportation of the protein to the correct cellular compartment, accurate post translational modifications and authentic antigenicity (Hruby, 1990; Moss, 1991). The lectin Concanavalin A, derived from the Jack bean, shows high affinity binding towards D-mannose sugars, found on glycoproteins (Hendricks *et al.*, 1988; Von Damme *et al.*, 1998). A method based on the ability of Concanavalin A to capture the G glycoprotein of HRSV was adapted for use with HMPV and subsequently used to screen and isolate anti-G glycoprotein hybridomas (Robinson, 2007).

Here, BALB/c mice were immunised with whole HMPV grown in 16HBE140 cells and screened by Concanavalin A ELISA using as antigen mouse L cells infected with recombinant vaccinia virus expressing the G glycoprotein. This was to increase the availability of the G glycoprotein for detection by monoclonal antibodies but also to reduce the non-specific binding of cross reactive 16HBE140 anti-host cell antibodies that would lead to false positives (Routledge *et al.*, 1985).

Intriguingly, screening hybridomas on vv174G5 and VTF7.3 created some difficulty attributable to certain hybridomas reacting with both positive and negative controls equally. This observation was strange since the immunised mice had not encountered vaccinia virus before and therefore should not have produced vaccinia virus specific antibodies. To circumvent this anomaly, a mixture of positive and cross reacting

hybridomas were picked and subsequently screened on antigen produced from HMPV infected 16HBE140 cells to eliminate non-HMPV secreting hybridomas.

Four anti-HMPV monoclonal antibodies were eventually isolated and tested by immunofluorescence, all of which showed strong staining when tested against HMPV174. Further analysis by immunofluorescence on recombinant vaccinia virus expressing HMPV F and G proteins revealed MAbAT1 to be an anti-G glycoprotein antibody whereas MAb2 and 4 were surprisingly anti-F monoclonal antibodies. Interestingly, the final antibody MAb5 was directed towards an internal HMPV protein deciphered by the staining pattern in HMPV174 infected cells. Despite initially screening on captured G glycoprotein using the Concanavalin A ELISA, three HMPV specific MAbs have been inadvertently generated that are not directed towards the G glycoprotein.

A possible explanation is that immunisation of mice with HMPV in 16HBE140 cells gave rise to antibodies which also bound the vaccinia virus VTF7.3 in L cells by ELISA. These antibodies are not HMPV specific as they were not recovered when selecting and cloning hybridomas on HMPV / 16HBE140 cells and furthermore, all hybridomas failed to react with VTF7.3 once cloned. Instead, these antibodies are most likely directed towards a host protein which is up-regulated by VTF7.3 infection and was not evident when the ELISA was being developed since the serum used for development had been absorbed with 16HBE140 cells.

Neutralisation analysis of all four antibodies revealed that the anti-F MAbs MAb2 and 4 have 50% neutralisation efficiency against HMPV174 at a concentration of approximately 17.7 $\mu$ g/ml and 0.13 $\mu$ g/ml, respectively with MAb4 comparable to that of Palivizumab at  $\sim$  0.5 $\mu$ g/ml (Johnson *et al.*, 1997) although interestingly, these antibodies were strain specific only showing definite staining with HMPV174. This is surprising given that the F glycoprotein is one of the most highly conserved proteins in HMPV with 94% amino acid identity between lineages (Biacchesi *et al.*, 2003; Boivin *et al.*, 2004; van den Hoogen *et al.*, 2004). The anti-G MAbAT1 was also strain specific which is not surprising given the G glycoproteins highly variable nature. Furthermore, the antibody did not neutralise suggesting either the antibody did

not have high enough avidity to coat the virion or its target was not a critical epitope required for attachment. MAbs AT1, 2 and 4 did not blot but could easily be identified as anti-G and anti-F antibodies by immunofluorescence on recombinant vaccinia virus. Generally, neutralising antibodies have greater requirements for the native epitope conformation suggesting the anti-F MAbs are probably directed towards a conformational type epitope (Ma *et al.*, 2005).

MAB5, the proposed anti-P antibody, which on the basis of western blot appeared to bind the viral phosphoprotein, did not neutralise which is not unanticipated since antibody directed towards an internal protein would not prevent virus attachment and entry into the host cell upon infection. Similar to MAb2 and 4, this antibody was strain specific which is again surprising given that the phosphoprotein displays more than 78% identity between lineages with up to 100% intra-lineage identity (Bastien *et al.*, 2003; Ishiguro *et al.*, 2004).

This antibody stained a HMPV specific band at around 38KDa when blotted with numerous lighter and heavier non specific bands. Previous reports have identified the phosphoprotein at approximately 40KDa (Biacchesi *et al.*, 2005a; Buchholz *et al.*, 2005), this along with the staining pattern observed by immunofluorescence would allow MAb5 to fit this profile.

## **8.9 Animal models**

The final aim of the project was to develop an animal model for the infection of HMPV that could be employed to complete a protection study. Since the discovery in 2001, a variety of species have been found to be permissive to HMPV infection. These include small animals such as mice, rats, hamsters and guinea pigs as well as primates including chimpanzees, cynomolgus macaques and African green monkeys (Alvarez *et al.*, 2004a; Darniot *et al.*, 2005; Hamelin *et al.*, 2005; Herd *et al.*, 2006; Kuiken *et al.*, 2004; MacPhail *et al.*, 2004; Skiadopoulos *et al.*, 2004; Williams *et al.*, 2005b; Wyde *et al.*, 2005).

Since previous studies within this laboratory with HMPV in BALB/c mice have proven successful (Robinson, 2007) these animals were used to study the infection of HMPV *in vivo*. Mice were subjected to various titres ranging from  $5 \times 10^5$  ffu/ml (passage 4), which caused a slight loss in weight yet infectious titres of  $10^{2.96}$  ffu/g and  $10^{2.49}$  ffu/g were recovered from the nasal mucosa and lungs respectively on day four post-infection, to  $7 \times 10^6$  ffu/ml (passage 12), where no infectious virus could be recovered yet mice were severely ill and lost up to 19% of their body weight as a result of infection. Along with weight loss, these mice exhibited breathing difficulties, ruffled fur and a tendency to huddle comparable to the observed pathogenesis of HMPV in other studies (Alvarez *et al.*, 2004a; Darniot *et al.*, 2005; Hamelin *et al.*, 2005).

These results contrast markedly with those reported by others where HMPV passaged in either Vero or LLC-MK2 cells in the presence of exogenous trypsin resulted in titres ranging from  $3.3 \times 10^5$  pfu/ml (Darniot *et al.*, 2005) up to  $10^8$  TCID<sub>50</sub> (Hamelin *et al.*, 2005). Hamelin *et al.* (2005) reported a comparable mean weight loss of 18.2% although they were able to recover peak viral titres of  $1.92 \times 10^7$  TCID<sub>50</sub> / g of lung on day five post-infection. Darniot *et al.* (2005), who also observed signs of disease including a mean weight loss of 17.2%, were able to recover infectious virus which peaked at day 4 ( $\log_{10} 2.37$  pfu/g) post infection. The discrepancies observed in these studies may reflect the differences in dose and virus strain which could alter the growth kinetics *in vivo*.

In HRSV-infected mice, to observe significant viral replication and clinical alterations, a high viral inoculum is required (Jafri *et al.*, 2004). However, the results from this study indicate that instead of increasing the recovery of infectious virus, an increase in titre (and passage level) had reduced viral replication in both the lungs and nasal mucosa although an increased weight loss was observed. This is contradictory to other reports where a dose-dependent relationship on the severity of infection was illustrated (Hamelin *et al.*, 2005).

Quantitative PCR confirmed the presence of viral RNA in the nasal mucosa and lungs of mice inoculated with  $2.6 \times 10^6$  ffu/ml (passage 11). There was evidence of viral replication which peaked at  $10^{4.17}$  in the nasal mucosa and  $10^{4.96}$  in lungs on day four

post-infection. However, no rise in viral RNA was observed when the inoculum increased to  $7 \times 10^6$  ffu/ml (passage 12) although RNA levels, presumably representing residual inoculum, exceeded  $10^6$  up to day four when the last mice was culled. Nonetheless, these results indicate that once the inoculum had been cleared, there was evidence of genome replication in both the lungs and nasal mucosa.

Despite the absence of infectious virus in the lungs or nasal mucosa of mice inoculated with HMPV174 passage 12 at a titre of  $7 \times 10^6$  ffu/ml, there was immunopathological evidence of limited viral protein synthesis. In addition, whilst there was some evidence of inflammation in the 16HBE140 infected mice, significant interstitial inflammation was apparent in the HMPV infected mice along with considerable areas of consolidation and destruction of alveolar walls. Taken together, these observations are suggestive of an abortive infection.

### **8.10 Does the G glycoprotein protect against challenge?**

The G glycoprotein of HRSV displays high levels of variability, similar to that observed in HMPV, thought to be attributed to the selective pressure of circulating antibodies (Cane *et al.*, 1991). Interestingly, whilst both G glycoprotein specific polyclonal anti-serum and pools of anti-G MAbs have displayed enhanced neutralisation, none of the anti-G monoclonal antibodies described to date have high neutralising activity (Martinez and Melero, 1998). These monoclonal antibodies were, however, effective in protecting mice and cotton rats against RSV infection (Taylor *et al.*, 1984; Walsh *et al.*, 1984).

Since the immunogenic and protective effects of the F glycoprotein of HMPV have been extensively studied (Skiadopoulos *et al.*, 2006; Skiadopoulos *et al.*, 2004; Tang *et al.*, 2005; Tang *et al.*, 2003), the ability of antibodies directed towards the HMPV G glycoprotein to protect against challenge was explored to establish a role *in vivo*. In addition to receiving subcutaneous immunisations, all animals were subjected to two intranasal inoculations of antigen delivered with cholera toxin adjuvant. Previous studies have shown that antigen administered by intranasal inoculation with *Escherichia coli* heat-labile toxin (LTB) together with trace amounts of cholera toxin

(CT) induced a high serum and secretory IgA antibody response with increasing titres upon secondary inoculations (Matsuo *et al.*, 2000; Tamura *et al.*, 1992; Tamura *et al.*, 1994). Protection in the upper respiratory tract correlates with high levels of IgA which operate independently from serum antibodies (Mills *et al.*, 1971; Prince *et al.*, 1987), whilst IgG is the predominant antibody found in the lungs and mediates protection in the lower respiratory tract (Crowe and Williams, 2003). However, upon evaluating pre-challenge antibody titres, there was no sign of detectable HMPV specific IgA response in any of the samples collected. The inability to also detect HMPV specific IgA in the serum of hyper immune mice indicated the assay was unsuccessful. Although titres were not comparable as different antigens were used, detection of serum IgG antibody titres by ELISA confirmed HMPV174 and vv174G immunised mice had specific HMPV antibodies. Further analysis of the serum antibodies revealed the anti-G specific polyclonal antibodies to exhibit high levels of neutralising activity similar to those observed with the monospecific anti-G antibodies.

Previous reports by Skiadopoulos *et al* (2006), where the G glycoprotein from HMPV was expressed in a recombinant PIV1 by reverse genetics, concluded that whilst immunisation with CAN97-83 (isolate of unknown passage propagated in LLC-MK2 cells) protected against challenge with homologous virus, the G glycoprotein induced only a weak protective response in hamsters. Protection was evaluated by a  $1.0\log_{10}$  or greater reduction in viral titres in either the lungs or nasal mucosa. In addition, despite the presence of HMPV-binding antibodies ( $7.5\log_2$ ), there was no induction of neutralising antibodies even after a second booster immunisation. In addition, work by Mok *et al* (2008) also concluded that despite the presence of elevated levels of HMPV-G specific antibodies, they were not neutralising and immunisation with virus replicon particles encoding the G glycoprotein did not confer protection against challenge in the animal model.

In this study, post challenge, quantitation of viral RNA illustrated that immunisation with whole virus protected the animals in both the upper and lower respiratory tract against challenge with the homologous strain. However, there was no significant difference in the quantity of viral RNA recovered from either the lungs or the nasal mucosa between vv174G and VTF7.3 immunised mice, corroborating previous

reports (Skiadopoulos *et al.*, 2006). However, detectable levels of neutralising antibodies were induced in the vv174G immunised mice. This result is surprising since other groups were unable to elicit neutralising antibodies directed towards the G glycoprotein (Mok *et al.*, 2008; Skiadopoulos *et al.*, 2006). This disparity suggests that either expression of the G glycoprotein in the vaccinia virus system has the ability to induce neutralising antibodies or potentially, the detection system for neutralising antibodies using 16HBE140 cells is more sensitive. It is, however, uncertain whether these antibodies are knocking out a key function or just coating the virus particle and preventing infection. If the former is true, then the failure of these antibodies to protect *in vivo* suggests that the G glycoprotein is not involved in infection of the mouse model. This differs from HRSV, where anti-G antibodies are protective via an Fc mediated mechanism (Mekseepralard *et al.*, 2006). In HRSV, it is thought that the role of the G glycoprotein is not in infection but subsequently the spread of the virus. Since infection of HMPV in this study appears to be abortive, antibodies are only being tested for their ability to block infection, not subsequent spread of the virus within the lung. It may be, therefore, that this animal model is not appropriate for testing the efficacy of anti-G immunity.

The inability of these antibodies to protect mice against challenge with the homologous virus suggests that the occupancy theory cannot fully explain our observations as antibodies coating the surface of the virions sufficient to prevent infection in cell culture should also prevent infection *in vivo*. Instead the anti-G antibodies appear to be neutralising by inactivating a specific epitope, which in cell culture is necessary for attachment and entry, but which is not required in the animal model. This suggests that this mouse model may not be reflecting the role of the G protein during human infection.

The significant loss of weight, post challenge, in mice immunised with HMPV174 was surprising since these animals were protected in both the lungs and nasal mucosa. There were no extensive differences in the histological data which could account for this observation. In similar experiments with mice immunised against RSV grown in cell culture and challenged with the same antigen, Piedra *et al* (1989a, 1989b) observed a severe lung response to the host cell element in the challenge inoculum. Here, immunisation of these animals with HMPV infected 16HBE140 cells could

have caused an analogous adverse reaction to the challenge material comprising of the same antigen. This wasn't observed in the recombinant vaccinia virus immunised mice which were not pre-exposed to the same immunogens having been immunised with recombinant vaccinia virus grown in mouse L cells.

### **8.11 SH genes**

Once cloned into pTM1, sequence analysis of the SH genes from HMPV128 passage 10 and HMPV174 passage 2 with sequences from the NCBI database revealed similar levels of variability to those reported by others (Biacchesi *et al.*, 2003; Ludewick *et al.*, 2005). There were relatively high levels of homology between members of the same sub-group, resulting in 91% nucleotide identity which was comparative to 88% reported by Biacchesi *et al.* (2003). A higher degree of variation was observed between sub-groups, where strains preserved 68-71% nucleotide homology (69% previously reported) (Biacchesi *et al.*, 2003).

During the initial cloning of the SH gene from HMPV174 passage 11 for the generation of a recombinant vaccinia virus, the gene was found to possess a nucleotide deletion at residue 64. This mutation resulted in a frame shift creating an ORF encoding only the 21 amino acids at the cytoplasmic tail of the SH. Hence, this frame shift ablates the expression of the majority of the SH protein. Intriguingly, this mutation was observed in the SH gene of HMPV145 at exactly the same position and the two genes were closely related with 99% nucleotide homology. Further examination of the B subtype SH gene from HMPV128 revealed there to be no such mutation at this residue. However, further downstream, a nucleotide substitution at residue 203 was observed resulting in the generation of a premature termination codon. This alteration would allow the cytoplasmic and transmembrane region to be translated with expression of approximately 10 amino acids in the luminal domain. Sequence analysis of the SH genes from earlier stocks (passage 2) of HMPV174 and 145 revealed both strains to possess the wild type phenotype. An earlier passage stock of HMPV128 was not available. Nonetheless, this suggests that these mutations had occurred as a result of passage in cell culture. Corroborating this observation was the sequence evaluation of two other A subtype isolates at passage 2 in cell culture, HMPV228 and 230, which were found to possess the wildtype phenotype.

Mutations of the small hydrophobic protein of HMPV have been observed elsewhere (Biacchesi *et al.*, 2007) where just over 50% of independent preparations tested possessed a nucleotide insertion at position 64. This mutation similarly resulted in a frame shift, ablating the expression of almost all of the protein. Other mutations, including point mutations and to a lesser extent nucleotide deletions, occurred less frequently but there was no observation of a nucleotide substitution at residue 203 comparable to the HMPV128 SH gene. Furthermore, Biacchesi *et al* (2007) suggested that each mutation arose beginning with a frame shift mutation since some populations contained only the frame shift whereas others possessed both the frame shift combined with additional mutations. The results obtained from our isolates however, do not support this notion since HMPV128 SH gene only possesses a nucleotide substitution with no evidence of insertions or deletions leading to a frame shift mutation.

It is well established that RNA viruses sustain mutations at a frequency of approximately  $10^{-4}$  per base, allowing the capacity for favourable variants to outgrow in response to selective pressure (Biacchesi *et al.*, 2007). The high frequency of truncations occurring in the SH gene suggests that the SH protein is not essential and may be detrimental for replication *in vitro*.

A study by Techaarpornkul *et al* (2001) with HRSV showed that in the absence of the G protein, virus expressing the F and SH proteins displayed somewhat smaller plaques, lower fusion activity and slower viral entry than virus expressing the F protein alone suggesting the SH protein has a dampening effect in cell culture. A similar phenomenon could be a possible explanation for the high frequency rate of mutations occurring the SH protein of HMPV.

## **8.12 Vaccinia virus expression of the SH glycoprotein of HMPV128**

Cells expressing the truncated SH gene from HMPV128 displayed moderate levels of staining when assessed by indirect immunofluorescence with the polyclonal HMPV128 anti-sera. However, there is evidence to suggest that even the functional SH glycoprotein is poorly expressed in wild type HMPV despite the presence of high

levels of SH mRNA and it might be unreasonable to expect bright fluorescence even with a good anti-serum (Skiadopoulos *et al.*, 2006). Reduced levels of reactivity could be attributed to the poor immunogenicity of the virus rather than the low abundance of protein produced in the expression system. Mice immunised with HMPV128 did not generate high titres of total HMPV antibodies and required a further immunisation compared with HMPV174 and 145. In addition, the anti-sera generated may only contain low levels of anti-SH antibodies as Connors *et al* (1991) have reported that the related HRSV SH glycoprotein, which like this truncated form of HMPV SH has a small luminal domain, is poorly immunogenic, perhaps as a consequence of heavy levels of glycosylation which might affect the antigenicity (Biacchesi *et al.*, 2004b). Expression of the functional SH gene from HMPV174 could not be detected by immunofluorescence with homologous polyclonal mouse anti-sera since HMPV174 expressing the mutated SH glycoprotein was used to generate the anti-sera.

### **8.13 Reactivity of monospecific anti-128SH glycoprotein sera**

Despite the stop codon at amino acid 68, the truncated form of HMPV128 SH expressed from recombinant vaccinia virus proved highly immunogenic. Interestingly, anti-SH monospecific antibodies directed towards HMPV128 were cross reactive with both HMPV174 and HMPV145. Both of these strains were in possession of a mutated SH protein where only the first 21 amino acids were expressed. However, the observation that the SH protein of HMPV128 possessed a nucleotide substitution at residue 203 resulting in the generation of a premature termination codon, suggest that the cross-reactive antibodies generated to this immunogen might be directed towards the transmembrane / cytoplasmic region of the protein and potentially the 14 amino acids expressed in the luminal domain. Conservation in this region would permit the induction of cross reactive antibodies with all three strains. Sequence analysis revealed there to be a 76% amino acid identity between sub-groups in the first 21 amino acids of the SH glycoprotein (data not shown) making this a feasible hypothesis.

No membrane staining was observed in HMPV128 infected cells where the antibody was made against the homologous construct. The same serum however did react in

membrane fluorescence with the full length HMPV174 SH glycoprotein. This suggests that the truncated HMPV128 SH protein is not actually incorporated and expressed on the surface of the infected cell and presumably, not the virion. Studies by Biacchesi *et al* (2007) involving the detection of a truncated form of the SH protein by Western blot analysis of purified virions with rabbit antiserum raised against a SH derived peptide (aa 82-96), revealed there to be no specific staining compared to wild type. These results were suggested to be attributable to the lack of incorporation of the alternative form of the SH protein into the mature virion (Biacchesi *et al.*, 2007).

#### **8.14 Neutralisation of HMPV by anti-128SH antibodies**

Despite the SH glycoprotein being non-essential for replication in cell culture, anti-SH128 antibodies were able to neutralise HMPV174 passage 2 but not HMPV128 passage 10. This suggests that the presence of the SH glycoprotein allows neutralisation, enabled by the antibodies ability to coat the virion and impede enough viral antigens as to inhibit viral attachment and entry. The inability of these antibodies to neutralise HMPV128 may be due to the different phenotypes of the two viruses. HMPV174 possesses the wildtype phenotype with membrane expression of SH. HMPV128 contains the mutated SH glycoprotein, expressing only the first 67 amino acids which were not expressed on the membrane. Sequence homology between the first 14 amino acids in luminal domain of the SH gene of HMPV174 and HMPV128, which represent the luminal amino acids expressed in the truncated form, is 92% and it is therefore likely that the antibodies generated to this luminal region of the truncated HMPV128 SH protein recognise and neutralise HMPV174.

However, studies by Skiadopoulos *et al* (2006), where recombinant human parainfluenza virus expressing the SH glycoprotein was evaluated in hamsters indicated this vector was not effective at inducing HMPV neutralising antibodies despite the addition of a booster immunisation. Furthermore, it appeared that the SH protein was only negligibly immunogenic and its protective effect was insignificant despite being much larger than its paramyxovirus counterparts.

### **8.15 Western blot analysis of anti-128SH antibodies**

Monospecific antibodies generated to the SH protein of HMPV128 displayed similar staining patterns to the monospecific anti-F and anti-G antibodies, however, there were several HMPV specific bands evident. These included bands at approximately 130KDa, 160KDa and above 170KDa all specific to HMPV174 passage 2, potentially representing the glycosylated forms of the protein as previously described (Biacchesi *et al.*, 2003; Ishiguro *et al.*, 2004). Identical staining was not apparent in the HMPV128 lane, as this virus possessed the mutated form of the SH protein with a premature termination codon. Furthermore, it is unlikely that the truncated protein would form high molecular weight glycosylated forms since there is only one serine present that could potentially act as an acceptor for O-linked glycosylation. The antibody also stained apparently HMPV specific bands at 14KDa and 12KDa possibly representing proteolytic cleavage products of the SH protein. This could have occurred since HMPV is a slow growing virus and requires at least 14 days in cell culture to reach full CPE. As a result, proteins could be partially digested during infection by host cell proteases despite the addition of protease inhibitors when preparing the viral antigens for blotting. It is not clear why fragments reacting with antibodies generated to the truncated form of the HMPV128 SH protein should not appear in HMPV128 infected cells.

### **8.16 Implications of the SH glycoprotein *in vivo***

The discovery that all working stocks of virus possessed the truncated form of the SH glycoprotein was unexpected and inadvertently all *in vivo* studies had been completed by this time. Consequently, all stocks used for the animal studies were sequenced and compared with genes already sequenced from the laboratory. Interestingly, all high passage stocks of both HMPV174 and HMPV145 contained only the mutated virus. However, the low passage stock used for the original growth curve contained a mixed population of both wild type and mutated virus. Furthermore, RNA from all *ex vivo* samples was extracted, sequenced and compared. Again, all samples from mice infected with high passage stocks of both viruses contained only the mutated form of the SH glycoprotein. However, samples from mice infected with passage 4, where a low titre of infectious virus was recovered from the lungs, despite the low titre

inoculum, contained a mixture of both wildtype and mutated virus. In addition, one sample from this experiment contained only wild type virus.

Biacchesi *et al* (2004b) developed a recombinant virus where the SH protein of HMPV has been deleted by reverse genetics allowing investigations into the effects of this mutant both *in vitro* and *in vivo*. Results indicated this mutant was readily recovered and was found to replicate efficiently in LLC-MK2 cells. Furthermore, somewhat more efficient replication in the lungs of hamsters was observed compared to wild type HMPV suggesting that the SH glycoprotein is completely dispensable *in vivo* and that its deletion does not confer attenuation in the rodent model (Biacchesi *et al.*, 2004b). A continuation of this work where the mutant was evaluated for replication in a primate model revealed replication of the virus was only marginally less efficient than that of wild type HMPV showing the protein is superfluous for efficient replication *in vivo* (Biacchesi *et al.*, 2005a).

Despite these reports, the possibility remains that the SH glycoprotein may have a crucial role in the efficient replication of the virus *in vivo* since every direct clinical isolate of HMPV described to date contains a completely functional SH gene and SH ORF (Ishiguro *et al.*, 2004; van den Hoogen *et al.*, 2002). Here, release of infectious virus correlated not with input virus titre but with the presence in the inoculum and recovery from infected tissues of virus carrying a wild type SH gene. These observations may not be incompatible with those of Biacchesi *et al* (2004b, 2005a) if the truncated form of the SH protein interferes with the production of infectious virus. If like the truncated form of HMPV128 SH, the truncated HMPV174 SH protein is not expressed on the surface of the virion, it may interfere with glycoprotein maturation in the endoplasmic reticulum producing an abortive infection. Why this effect should occur *in vivo* but not in cell culture is not clear. As Biacchesi *et al* deleted the entire protein, this was not evident in their study. In addition, there is a possibility that the SH gene mutation is an incidental factor and that other mutations may have occurred on passage in cell culture which produce an abortive infection *in vivo*.

The discrepancy between the results from this study and those obtained in other laboratories studying virus adapted to culture in monkey kidney cells in the presence of exogenous trypsin are striking.

Firstly, HMPV grown in 16HBE140 cells without exogenous trypsin was neutralised by monospecific antibodies to all three surface glycoproteins, whilst monkey kidney cell adapted virus was neutralised by only anti-F antibodies. Secondly, high passage virus with a truncated SH does not replicate *in vivo*, suggesting the SH is essential for *in vivo* infection. Thirdly, relatively low titres of virus produced serious weight loss in the mice with many cells infected. This may be due to the ability of 16HBE140 cells to cleave the virus on exit and produce virus capable of more efficient infection in the mouse lung.

## 8.17 Conclusions and future work

This study has highlighted marked differences between high and low passage virus and the dissimilarities associated with the use of different disease models. The inability of the G glycoprotein alone to protect in the mouse model is surprising given its extensive level of variability reported throughout the sub-groups. High concentrations of mutations, especially in the ectodomain, is an indicator that this protein is under immunological pressure suggesting it has an important function which is essential for infection in the human host. Therefore it is essential to determine its biological function. The observation here that this virus can readily mutate to lose an entire surface glycoprotein, SH, *in vitro*, would suggest HMPV would readily ablate the expression of the G protein if not indispensable for infection and replication.

Furthermore, the ability of the G glycoprotein to induce neutralising antibodies might be construed to indicate a potential role for the protein in attachment or entry of the virus into the host cell. The inability of these antibodies to protect *in vivo* but neutralise *in vitro*, suggests a potential flaw in the animal model and indicates the requirement for further work to establish the differences between the two systems.

That mutations arise in the SH glycoprotein on passage in cell culture, that ablate the expression of almost the entire protein is consistent with studies in which the SH protein has been proven to be completely dispensable for infection both *in vitro* and *in vivo*. Yet every single direct clinical isolate described to date possesses the wildtype phenotype. Furthermore, the ability of antibodies directed towards the SH glycoprotein, prepared in this study, to neutralise HMPV in 16HBE140 cells, may imply it has a function in either the attachment or entry into the host cell, acting alone or in combination with the other two glycoproteins.

In addition, the correlation of SH mutation and inability to replicate *in vivo* on passage suggests this protein has an important role in modulating the spread of the virus in the lung. However, the work presented here was limited by the failure to passage the virus to high titres without it mutating. This meant low passage stocks would be unable to

establish an infection *in vivo*. Therefore, further work is required to preserve the wild type form of the virus in cell cultures yielding sufficient virus for *in vivo* studies.

Finally, the apparent heterologous nature of HMPV145 with genes related to both genotypes A2 and B1 requires further investigation. Genes need to be amplified across the intergenic region allowing the incorporation of two genes in one amplicand to determine whether this virus derives from a mixed infection or is a true recombinant.

The function of the SH glycoprotein and the G glycoprotein remains elusive. It is hypothesised that these glycoproteins are more than just accessory proteins and the assumption that the F glycoprotein is the only major protective antigen is naïve in view of high levels of variability of the two proteins in the wild. The development of further model systems which may more closely resemble the conditions in the human respiratory tract and an increased cognizance of the changes which occur when viruses are passaged in non human hosts may be necessary to resolve these issues.

## Chapter 9: References

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## **Appendix 1**

### **A.1 F sequence**

#### **A.1.1 NCL03-04/128**

Sequenced with forward and reverse primers where bases 455 to 1009 were a consensus of the two sequences. Bases 1618 to 1620 represent the F gene stop codon.





### **A.1.2 NCL04-04/145**

Sequenced with forward and reverse primers where bases 461 to 1089 were a consensus of the two sequences. Bases 1618 to 1620 represent the F gene stop codon.





### **A.1.3 NCL03-04/174**

Sequenced with forward and reverse primers where bases 623 to 957 were a consensus of the two sequences. Bases 1618 to 1620 represent the F gene stop codon.





## **Appendix 2**

### **A.2 G sequence**

#### **A.2.1 NCL03-04/128**

Sequenced with forward and reverse primers where bases 60 to 696 were a consensus of the two sequences. Bases 694 to 696 represent the G gene stop codon.



### **A.2.2 NCL03-04/145**

Sequenced with forward and reverse primers where bases 46 to 732 were a consensus of the two sequences. Bases 730 to 732 represent the G gene stop codon.



### **A.2.3 NCL03-04/174**

Sequenced with forward and reverse primers where bases 48 to 654 were a consensus of the two sequences. Bases 652 to 654 represent the G gene stop codon.



## **Appendix 3**

### **A.3 SH sequence**

#### **A.3.1 NCL03-04/145 (1-752)**

Sequenced with forward and reverse primers where bases 54 to 690 were a consensus of the two sequences. Bases 549 to 551 represent the SH gene stop codon.



### **A.3.2 NCL03-04/174 (1-752)**

Sequenced with forward and reverse primers where bases 31 to 701 were a consensus of the two sequences. Bases 549 to 551 represent the SH gene stop codon.



### **A.3.3 NCL03-04/228 (1-752)**

Sequenced with forward and reverse primers where bases 61 to 701 were a consensus of the two sequences. Bases 549 to 551 represent the SH gene stop codon.



#### **A.3.4 NCL03-04/230 (1-752)**

Sequenced with forward and reverse primers where bases 53 to 704 were a consensus of the two sequences. Bases 549 to 551 represent the SH gene stop codon.



## **Appendix 4**

### **A.4 N sequence**

#### **A.4.1 NCL03-04/145 (35-197)**

Sequenced with forward and reverse primers.