The role of genotype specific anti-G antibodies in protection against severe human respiratory syncytial virus infection

Cheng Siang Tan

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Abstract

Human respiratory syncytial virus is a leading cause of lower respiratory tract infection in infants. Current prophylaxis with Palivizumab, a humanised anti-fusion glycoprotein monoclonal antibody, has only achieved moderate success. In animal models, immunization with G glycoprotein has led to largely genotype specific immunity. This suggests the hypothesis that anti-G antibodies to the infecting virus genotype, either acquired transplacentally or induced by immunisation, may contribute to protection of the infant in the early months of life.

The aim of this study has been to test this hypothesis by measuring levels of maternally acquired antibody to the G glycoprotein of the infecting virus genotype in an index group of infants with severe HRSV infection and, to the same genotype, in a comparison group of infants with no history of HRSV infection. As only 2% of infants suffer severe disease on primary HRSV infection, the comparison group are assumed to be destined to be resistant to severe infection. A deficiency of anti-G antibodies in the index group will thus indicate a protective role for these antibodies.

A phylogenetic study was carried out in Newcastle upon Tyne from autumn 2007 to spring 2010 revealing three circulating genotypes of HRSV, namely GA2, GA5 and BA4 although GA5 was not detected in the third epidemic. The G genes of GA2 and BA4 and the F gene of GA2 were cloned and expressed individually in modified vaccinia Ankara virus. The recombinant proteins were used in the development of a concanavalinA capture ELISA sufficiently sensitive to detect maternal antibodies in the acute sera of infants under 6 months of age. An attempt was made to refine the assay in order to separately detect antibodies to the conserved and variable epitopes of the G glycoprotein. However, mapping of conserved and variable epitopes revealed overlap epitopes precluding the development of a differentiation in a simple ELISA assay.

An index group of infants with severe HRSV infection and a comparison group of infants of similar age with no history of HRSV infection were recruited with consent by their legal carer during the epidemic of 2009/2010. The infecting virus lineage of each infant in the index group, either GA2 or BA4, was identified by reverse transciptase-polymerase chain reaction of virus recovered from nasal secretions. Sera were collected from both groups, at the acute stage of infection for the index group, and screened for

HRSV specific IgA by ELISA. Only those without detectable IgA were included in the study.

IgG maternal antibodies to the recombinant G glycoprotein of the infecting virus genotype and the F glycoprotein of the GA2 genotype were measured in the sera of index and comparison group infants whilst maternal antibody levels to both F and G glycoproteins in the sera of both index and control sera decayed at similar rates with age, the index group possessed significantly more anti-G and anti-F antibody at all ages suggesting that infants hospitalized with severe HRSV infection were not deficient in antibodies to either glycoprotein contrary to the hypothesis under test. However, mean maternal antibody levels at birth have been shown to fall across the winter epidemic and the above analysis may be susceptible to bias introduced by uneven sampling of infants across the epidemic. To avoid this potential error, anti-F/anti-G antibody ratios, which give a measure of anti-G immunity independent of age and time of collection, were also compared in the sera of index and comparison group infants. Mean ratios were not significantly different between the two groups also rejecting the hypothesis that severely affected infants have a deficiency in maternal anti-G antibodies. These studies, therefore, fail to establish a role for anti-G glycoprotein antibodies in the protection of infants against severe lower respiratory tract disease.

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Standard abbreviations

aa	amino acid
Ag	antigen
AHR	airway hyper-responsiveness
APS	ammonium persulphate
BA	Buenos Aires
BB	streptococcal protein G
BCB	bicarbonate coating buffer
BCB	bicarbonate coating buffer
Bgal	beta-galactosidase
BMT	bone marrow transplant
BN	Bavarian Nordic
bp	base pair
BPD	bronchopulmonary dysplasia
BRSV	bovine respiratory syncytial virus
BudR	bromodioxyuridine
C-	carboxy-
CCA	chimpanzee coryza agent
cDC	conventional dendritic cell
cDNA	complimentary DNA
CEF	chicken embryo fibroblast
CF	complement fixation

ConA	concanavalin A
cp	cold passage
CPE	cytopathic effect
Crm1	nuclear export receptor
CTL	cytotoxic T lymphocyte
DC	dendritic cell
DEPC	diethyl pyrocarbonate treated water
DNA	deoxyribonucleic acid
dNTP	dideoxyribonucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
EtBr	ethidium bromide
F	fusion glycoprotein
FI	formalin inactivated
G	attachment glycoprotein
GAG	glucosaminoglycan
GM	growth medium
H	histidine
HeLa	human cervical carcinoma
Hep-2	human epithelial cell
HI	heat inactivated

HIV	human immunodeficiency virus
HMPV	human metapneumovirus
HPA	Health Protection Agency
HRA	heptad repeat region A
HRB	heptad repeat region B
HRSV	human respiratory syncytial virus
HSE	herpes simplex encephalitis
ICU	intensive care unit
IF	immunofluorescence
IFA	immunoflourescent assay
Ig	Immunoglobulin
IG	immunoglobulin
IKK	inhibitor of kappa kinase
IL	interleukin
IMP	inosine-5’-monophosphate
INF	interferon
IV	intravenous
KB	HeLa derived cell line
L-	levo
L	polymerase
LB	luria bertani
LBA	luria bertani agar

LCMV	Lymphocytic Choriomeningitis
Le	leader sequence
LRTI	lower respiratory tract infection
M	matrix
M	molarity
m	membrane
M	methionine
M	molarity
Mab	Monoclonal antibody
MCP	Macrophage chemoattractant protein
MCS	multi cloning site
MDA	melanoma differentiation association gene
MIP	Macrophage inflammatory protein
MM	maintenance medium
MOI	multiplicity of infection
MPA	monophosphoryl lipid A
mRNA	messenger RNA
MVA	modified vaccinia Ankara
N	nucleoprotein
N	nucleoprotein
N-	amino-
N	normal

NEAA	non-essential amino acids
NES	nuclear export signal
NK	Natural killer
NPS	nasopharyngeal secretion
NS	non-structural
NS	nose swab
NTD	amino terminal domain
OPD	Ortho-phenylendiamine dihydrochloride
ORF	open reading frame
P	Passage
p.i.	post infection/post inoculation
PAGE	polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBST	PBS-tween20
PBSTx	PBS-triton X-100
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cell
PFP	purified fusion glycoprotein
PRR	pattern recognition receptor
QT35	Quail Japanese fibrosarcoma
r	recombinant
RANTES	regulated upon activation, normal T-cell expressed, and secreted

RIG-I	retinoic acid-inducible gene 1 protein
RNA	ribonucleic acid
rpm	revolution per minute
RT	reverse transcription
s	soluble
SAF	serum and antibiotic free
SCID	severe combined immune deficiency
SDS	sodium dodecyl sulphate
SH	short hydrophobic glycoprotein
SNP	single nucleotide polymorphism
SOB	super optimal broth
SOC	super optimal broth with catabolic repression
SP	surfactant protein
STAT2	signal transducer and activator of transcription 2
TBE	tris boric acid EDTA
TBS	tris buffered saline
TEMED	N,N,N’,N’-tetramethylethylenediamine
Tfb	transformation buffer
Th	T helper cell
TK	thymidine kinase
TLR	toll like receptor
TNF	tumour necrosis factors

TPB	tryptose phosphate broth
Tr	trailer
TRAF	TNF receptor associated factors
ts	temperature sensitive
URTI	upper respiratory tract infection
V	volt
Vero	green monkey kidney epithelial cells
W	tryptophan
W	watt
wt	wild type
K	lysine
Y	tyrosine
I	isoleucine
P	proline
Q	glutamine
S	serine
F	phenylalanine
E	glutamic acid
H	histidine
A	alanine
L	leucine
V	valine

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Chapter 1 Literature Review

1.1 Origin

A novel virus was isolated from a colony of chimpanzees suffering from coryza in 1956 and named chimpanzee coryza agent (CCA). Following the discovery, antibodies to CCA was detected in the serum of a laboratory worker suffering from a cold, providing evidence that CCA or CCA-like agents were capable of crossing species barrier from chimpanzee to human. This was further confirmed with the isolation of CCA-like viruses from 2 infants with bronchopneumonia and laryngotracheobronchitis with respiratory tract infections named Long virus and Synder virus respectively (Chanock *et al.*, 1957) both Long virus and Synder virus exhibited identical cytopathogenic effect (CPE) with CCA virus which was described as the formation of syncytial areas although round cell degeneration also occurred in KB cells. A population study revealed that, by the age of 4 years, 80% of the children had been exposed to this infecting agent by the presence of neutralizing antibodies to the Long virus. Thus the these agents were grouped together and the term respiratory syncytial virus was introduced (Chanock and Finberg, 1957).

1.2 Classification and morphology

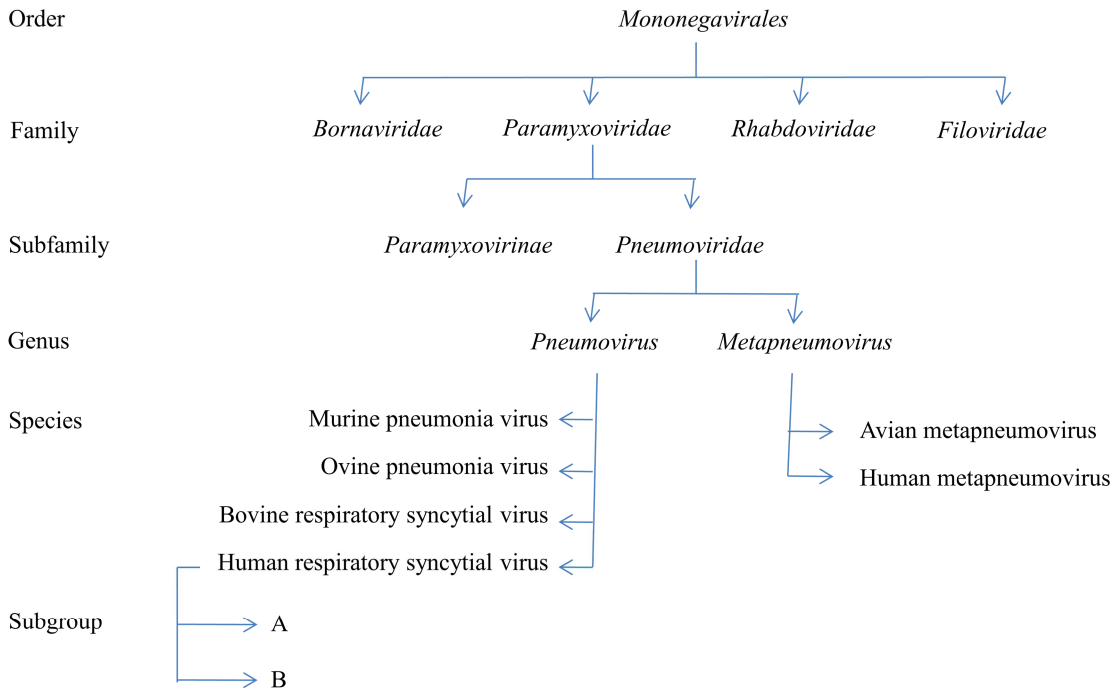

Figure 1 The taxonomy of HRSV

Adapted from The International Committee on Taxonomy of Viruses (ICTV).

The HRSV is the prototype strain of the genus pneumovirus within the *Paramyxovirinae* subfamily as shown in Figure 1. HRSV contains two serologically distinct subgroups, A and B which cocirculate during HRSV epidemics. Other members include bovine respiratory syncytial virus, ovine pneumonia virus and murine pneumonia virus. Within the subfamily is the genus Metapneumovirus which contains avian metapneumovirus and the recently found human metapneumovirus (HMPV) (van den Hoogen *et al.*, 2001). The clinical symptoms caused by HMPV ranges from upper respiratory tract infection to severe bronchiolitis and pneumonia, indistinguishable from the clinical symptoms caused by HRSV(van den Hoogen *et al.*, 2001).

The HRSV is a Baltimore Class V virus which contains a single-strand, non-segmented, negative sense RNA with the molecular weight of approximately 5.0 x 10^6Da (Y. T. Huang and Wertz, 1982). The actual size of the genome is strain dependent but the typical length is about 15k nucleotides (nt).

The HRSV virions are either spherical, filamentous or pleomorphic particles. The spherical particles are about 90-130nm in diameter (Norrby *et al.*, 1970) but particles with the diameter between 150-500nm have also been observed. (Bachi and Howe, 1973). Filamentous particles are predominantly observed having diameters ranging between 100 to 120nm. They can be as long as 10µm in length.

Peripheral spikes with the length of 10nm can be observed in all of the particles comprising one or all of the three transmembrane viral envelope proteins: the attachment (G), the fusion (F) and the small hydrophobic (SH) glycoproteins.

1.3 Genome, transcription and replication

The genome of HRSV is neither capped nor polyadenylated and carries 10 viral genes (P L Collins *et al.*, 1984a; P. L. Collins *et al.*, 1986) as shown in Figure 2. Genes are separated from one another by intron-like regions called the intergenic regions which vary in length from 1 to 53nt without any significant conservation (P. L. Collins *et al.*, 1986).

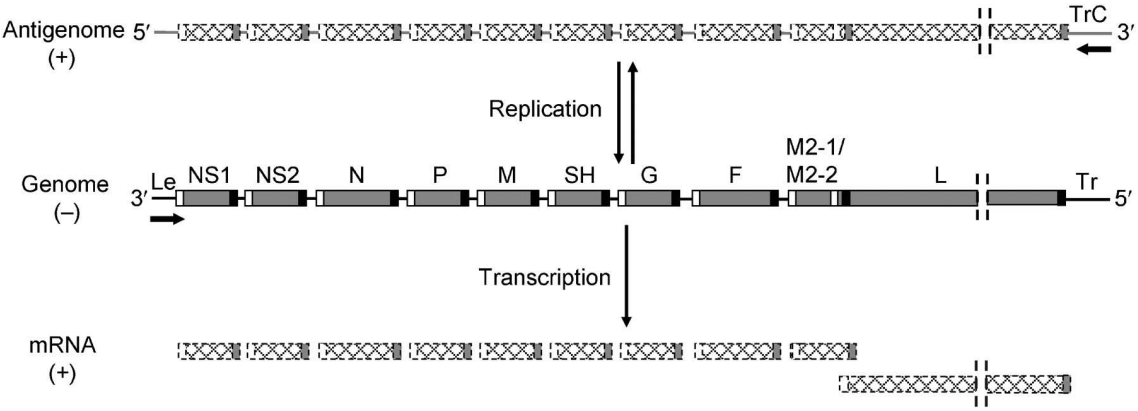

Figure 2 Diagram depicting the RSV genome and its transcription and replication products.

The virus genes are depicted as grey rectangles; the L gene, which comprises almost half of the genome, has been truncated. The GS and GE signals are shown as white and black boxes, respectively. The encoded antigenome and mRNAs are indicated by hatched rectangles. Arrows indicate the location of the promoters (Cowton *et al.*, 2006).

Each gene is flanked by the gene start (GS) and gene end (GE) sequences which direct the polymerase during transcription. The GS sequence for the first nine genes is conserved except the GS for the L gene. The function of each GS and GE is independent of the gene it flanks and can be attached to foreign genes to direct transcription into subgenomic mRNA (Kuo *et al.*, 1996). At the 3'- and 5'- ends of the genome are short extragenic regions known as the 44nt leader sequence (Le) and a 155nt trailer sequence (Tr) respectively (P. L. Collins *et al.*, 1991; Mink *et al.*, 1991).

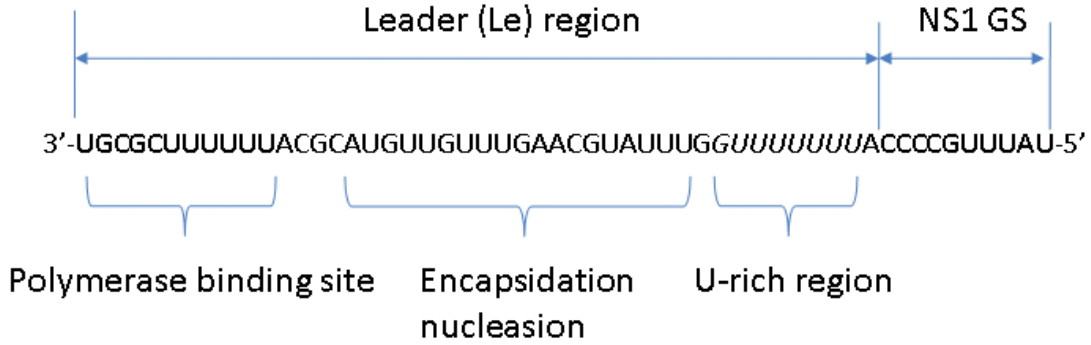

Figure 3 The negative sense RNA genomic sequence of Le and NS1 GS of HRSV.

Adapted from (McGivern *et al.*, 2005; Cowton *et al.*, 2006), based on strain A2. The polymerase binding site, U-rich region and region important for encapsidation is shown.

HRSV is monocystronic in nature with the sole promoter for transcription located in the Le where the polymerase binds to nt 1-11 and moves along the length of the genome

(Cowton and Fearns, 2005). There is an uracyl-rich (U-rich) region at the end of the Le sequence (Figure 3), just before the NS1 GS which determines the efficiency of transcription. The U-rich and NS1 GS sequence can tolerate up to 80nt foreign nucleotide insertion upstream without any significant inhibitory effect to transcription. However, deletions in the sequence upstream of the U-rich region cause inhibitory effects on transcription (Fearns *et al.*, 2000; McGivern *et al.*, 2005). The NS1 GS sequence is part of the sequential-transcription system (Cowton *et al.*, 2006) whereby mutation to this sequence causes up to 90% reduction of downstream genes transcript (Kuo *et al.*, 1996).

The GS sequence is believed to hold the template-bound polymerase for a sufficient time to allow the initiating nucleotides to enter the polymerase active site and to initiate RNA synthesis (Cowton *et al.*, 2006). When the polymerase reaches the GE sequence, it polyadenylates and releases the nascent mRNA. It then reinitiates RNA synthesis at the next GS sequence and caps (Liuzzi *et al.*, 2005) and methylates the 5'-of the RNA. Thus, the GS and GE signals help to produce polycistronic mRNAs for individual protein translation (P. L. Collins and Wertz, 1983). The GE sequence contains the UCAAU motif which is conserved in nine of the ten GE sequences. The GE motif is probably designed to be of moderate efficiency to ensure polymerase readthrough during transcription (P. L. Collins and Wertz, 1983) resulting in read through mRNAs in HRSV infected cells.

The M2-1 protein helps to enhance processivity of the polymerase which in turn ensures read through at the GE sequence (Sutherland *et al.*, 2001) and prevents inappropriate dissociation at the U-rich intergenic region (Hardy and Wertz, 1998; Sutherland *et al.*, 2001).

With sufficient processivity and GE read through capability, the polymerase will still have the tendency to dissociate from the genome resulting a gradient of expression, with the gene at the 3'- end being transcribed more frequently compared to those at the 5'-end of the genome (Krempl *et al.*, 2002). Thus, proteins that are required in vast quantity are positioned near the 3'- end while those needed in minute quantity are near the 5'-end (P. L. Collins and Crowe, 2001).

The polymerase has a dual function in transcription (as transcriptase) and replication (replicase) but the actual mechanism controlling these two functions is not yet

understood. During replication, the polymerase binds to the Le region and initiates full length antigenome synthesis from the 3'-end disregarding the GS and GE motifs. The first 34nt of the Le region are important for antigenome synthesis but the polymerase binding site remains the same as during transcription revealing a dual function for the first 11nt. The central region of the Le sequence (nt 16-34) is believed to mediate encapsidation nucleation of the antigenomic RNA and to ensure readthrough at the GE sequences (McGivern *et al.*, 2005)

At the end of the antigenome (positive strand genome) is the complement for the trailer (TrC) (Figure 2) which contains a promoter to generate the negative sense genomic RNA at a significantly higher level of replication than the Le resulting in higher concentration of negative sense genomic RNA (product of TrC promoter) compared to positive sense antigenomic RNA (product of Le promoter) in infected cells (Mink *et al.*, 1991).

The first 36nt in TrC is sufficient to initiate replication and to direct transcription when positioned upstream to a GS sequence. TrC and Le promoters have identical strength when the Le sequence was replaced with the TrC sequence in a minireplicon experiment. However, then the length of the introduced TrC sequence was increased to 147nt, replication was increased four-fold with almost total silencing of transcription suggesting that there is a non-essential cis-acting element which enhances replication. This element is thought to reside in nucleotide residues 36-97 but the exact location and mechanism has not been mapped to date. However, this cis-acting element is believed to accelerate polymerase binding to the promoter and speed up initiation of RNA synthesis and promoting encapsidation of the nascent RNA which might facilitate replication processivity (Fearns *et al.*, 2000). The genomic RNA is encapsidated with nucleoprotein immediately upon synthesis (Cowton *et al.*, 2006).

1.4 Viral proteins

1.4.1 Non-structural proteins (NS)

HRSV encodes for 2 nonstructural proteins from 2 open reading frames producing NS1 and NS2. They are synthesized in high quantity in infected cells but are not packaged into the matured virions (Bitko *et al.*, 2007).

NS proteins act as host range determinants. Bovine respiratory syncytial virus (BRSV) and HRSV grow in an identical manner in both human and bovine cells lacking intact interferon (INF) functions. However, when grown in INF competent human cells, the growth of BRSV was retarded and *vice versa*. Similarly, the growth of HRSV was insensitive to exogenous INF when grown in human cell line but growth was retarded by exogenous INF when grown in cells from bovine origin. (Bossert and Conzelmann, 2002).

The nonstructural proteins function by inhibiting host innate immunity especially the type I interferon (IFN) to ensure uninhibited growth in the host cells with NS1 playing a greater role compared to NS2 (Spann *et al.*, 2004). NS1 and NS2 are functionally related and were shown to form a heterodimer in immunoprecipitation experiment but can function individually or in concert in the inhibition of the IFN synthesis pathway and IFN function. (Swedan *et al.*, 2009). The deletion of NS1 and M2-2 caused attenuation of the virus in a primate model (Teng *et al.*, 2000) and severely retarded the viral replication, producing smaller plaques in Vero cell culture. NS2 is relatively unstable with the half life of 30 minutes suggesting that it plays an important role in the early phase of the life cycle. (Evans *et al.*, 1996).

NS2 alone can inhibit the INF production mediated by the retinoic acid induced gene I (RIG-I) and Toll-like receptor 3 (TLR3) pathways (Ling *et al.*, 2009). It also reduces the level of signal transducer and activator of transcription 2 (STAT2), a transcription factor for INF-inducible antiviral genes by proteasome-dependent degradation but increases the level of inhibitor of NF-kappaB kinase ε (IKKε) (Swedan *et al.*, 2009). However, NS1 is known to reduce the production of IKKε (Swedan *et al.*, 2009) suggesting a negative regulatory effect on NS2.

Both NS1 and NS2 work together in reducing the level of TRAF3 (Tumour Necrosis Factor receptor-associated factor 3) with NS1 playing a greater role (Swedan *et al.*,

2009). TRAF3 functions downstream of multiple (Tumour necrosis factor) TNF receptors and receptor that induces INF-α, INF-ß and INF-λ synthesis, including Toll-like receptor 3 (TLR3) (Pérez de Diego *et al.*, 2010) and is a negative regulator for NF-KappaB activation (Ikeda *et al.*, 2010). The genetic deficiency in TRAF3 ablates the ability of individuals to activate INF upon stimulation and is associated with increased susceptibility to herpes simplex virus-1 encephalitis (HSE) (Pérez de Diego *et al.*, 2010). The expression of both NS proteins prevent the early apoptosis of infected cells (Bitko *et al.*, 2007).

Although NS1 is not packaged into the matured virion, it might be involved in the virion packaging process as it was shown to be associated with the matrix protein in a co-immunoprecipitation experiments (Teng *et al.*, 2000). The absence of NS1 reduces the efficiency of the virion assembly and budding (Jin *et al.*, 2000).

1.4.2 **NPL complex**

The HRSV nucleoprotein (B. Garcia-Barreno *et al.*, 1996), phosphoprotein, polymerase (Khattar *et al.*, 2001) and M2-1 (Mason *et al.*, 2003) proteins work in concert in RNA synthesis.

Nucleoprotein (N)

Common with all other members of *Mononegavirales* order, HRSV encodes for a nucleoprotein, in this case of 391 amino acids (aa) in length,which forms a nuclease resistant helical ribonucleoprotein complex with the genomic RNA forming the nucleocapsid (P. L. Collins and Crowe, 2001). N forms a decameric ring like structure with each nucleoprotein subunit held together by van der Waals interaction between the highly hydrophilic N-terminal domain (aa36-253) and C-terminal domain (aa257-360). The decameric structure is further stabilised by the genomic RNA belt which runs within a basic surface groove surrounding the periphery of the ring with every 7 nucleotides interacting with one N subunit (Tawar *et al.*, 2009).

The C-terminal end of N was shown to bind to the phosphoprotein using monoclonal inhibition assay and peptide interference assays (Murray *et al.*, 2001).

With the resolution of the crystal structure of N, it was postulated that the polymerase can read along the RNA without the need to dissociate N from the genomic RNA by

transiently moving the N-terminal domain (NTD) like a lever exposing the bases during transcription (Tawar *et al.*, 2009). Encapsidation of the genomic RNA prevents the formation of secondary structure, eliminatinig the need of helicase and reduces the formation of double stranded RNA to avoid triggering host's antiviral response (Le Mercier *et al.*, 2002).

Phosphoprotein (P)

The phosphoprotein is a 241 amino acid protein which is folded into oligomer of ~500 kDa, possibly a homotetramer. It contains a central structured domain (aa100-200) flanked by two essentially disordered regions (aa1–99 and aa201–241) (Llorente *et al.*, 2006). P is heavily phosphorylated in Ser232 and the absence of phosphorylation at this site yielded a partially synthesized RNA transcript suggesting that phosphorylation is essential in the activation or stabilization of the polymerase (Dupuy *et al.*, 1999).

RNA dependent RNA polymerase (L protein)

The RNA dependent RNA polymerase is the largest protein encoded by HRSV which gives it the abbreviation L protein. The L gene of HRSV strain A2 is 6578nt long encoding a protein of 2165aa with a molecular weight of 250kDa as determined by electrophoretic mobility (Stec *et al.*, 1991; P. L. Collins and Crowe, 2001). The L protein sequence is rather similar to that of other paramyxoviruses but with a 70 aa extension at the N-terminus which is probably due to the adoption of a new translational start codon which overlaps with the upstream M2 gene. The N-terminus half of the L protein shares 20% homology with the equivalent regions of other negative stranded virus polymerases and contain 6 isolated segments that are colinear and highly conserved with 3 segments match sequence motifs also found in other RNA-dependent RNA and DNA polymerases (Stec *et al.*, 1991).

1.4.3 **Matrix protein (M)**

The matrix protein association with the NS1 protein suggests that it requires the NS1 for maturation, assembly (Teng *et al.*, 2000) and are responsible for connecting the viral membrane to the nucleocapsid and plays a role in RNA regulation (Ghildyal *et al.*, 2006). The M protein was shown to translocate into the nucleus of infected cells through the action of importin β1 nuclear import receptor and exit into the cytoplasm through the nuclear export receptor Crm1 directed by the nuclear export signal (NES) located in residues 194-206 of the M protein. Mutation of NES in recombinant HRSV resulted in reduced virus production (Ghildyal *et al.*, 2009).

The mRNA coding the M2 protein contains two overlapping open reading frames (ORFs) ORF1 encodes the 22kDa structural protein, M2-1 and ORF2 encodes a 10kDa protein (M2-2) (Hardy and Wertz, 1998).

M2-1

The M2 gene of HRSV encodes for a protein of 194aa in length with the molecular weight of 22kDa, thus previously known as the 22k protein. The M2-1 proteins coexist in 2 isoforms and can be distinguished by western blot whereby a 24kDa species can be observed (Routledge *et al.*, 1987). The 24k species is phosphorylated at Ser58 and Ser61 by casein kinase I (Cartee and Wertz, 2001). Interestingly the 24k species dominates when expressed alone but the 22k species dominates when co-expressed with other HRSV components. M2-1 self assembles into oligomers, possibly tetramers in infected cells (Tran *et al.*, 2009), a phenomenon which was initially described 2 decades ago when resolving the M2-1 through polyacrylamide gel in non-reducing condition (Routledge *et al.*, 1987).

The M2-1 protein serves as a transcription factor which increases polymerase processivity, and acts as a transcription anti-terminator by promoting readthrough of intergenic junctions during virus transcription (Hardy and Wertz, 2000). The M2-1 protein contains a Cys_3-His_1 motif (C-X_7-C-X_5-C-X_3-H) near the N-terminus and this pattern is conserved in all human, bovine, and ovine RSV. This motif, known as the zinc finger motif is known to bind zinc in another molecule and maintains the integrity and function of M2-1. It is believed that the binding of zinc to this site alters the 3 dimensional structure of M2-1 making it more accessible to cellular kinase for phosphorylation. Site directed mutagenesis experiments ablating the binding site of M2-

1 with the nucleocapsid have shown that M2-1 can function alone (Hardy and Wertz, 2000).

M2-2

Transcription of M2-2 is initiated by any of the three start codons upstream of the stop codon of M2-1 with the first two being more important. Studies have shown that the location of the M2-1 termination codon is crucial in regulating the translation of M2-2 suggesting that M2-2 is accessed by ribosomes that are leaving the ORF1 (Gholamreza Ahmadian *et al.*, 2000).

M2-2 is expressed as a separate protein from M2-1 but its expression is believed to be highly regulated by M2-1 (Cheng *et al.*, 2005) as the concentration of M2-2 is relatively low compared to that of other viral proteins (G. Ahmadian *et al.*, 1999).

M2-2 was initially found to be inhibitory to RNA transcription and transcription by RSV minireplicons and overexpression of the M2-2 protein completely inhibited the RSV replication (Cheng *et al.*, 2005). However, recent studies have shown that M2-2 acts as a regulatory factor for the RNA replication and transcription of HRSV in infected cells rather than being solely inhibitory. Experiments with the M2-2 knockout mutant (ΔM2-2), where the second start codon of the M2 gene is silent show that M2-2 increases RNA replication and regulates transcription. M2-2 promotes transcription in the early stage post infection and allows the mRNA to accumulate to a certain concentration. M2-2 downregulates transcription and upregulates replication when the threshold mRNA concentration is reached to favour genomic RNA synthesis. The absence of M2-2 attenuates the virus by the factor of 10^3, accelerates cytopathology *in vitro* but somehow increases gene expression. The deletion of the M2-2 can be an useful feature in vaccine design as it allows infection of the respiratory tract at low MOI but with the enhancement of viral protein production (Bermingham and Collins, 1999).

1.4.4 **Surface glycoproteins**

HRSV expresses three glycoproteins which are displayed on the surface of the infected cells. They are the small hydrophobic protein (SH), the attachment protein (G) and the fusion protein (F).

The small hydrophobic (SH) protein

The small hydrophobic protein is a 64-65aa long depending on strain (64aa for subgroup A and 65aa for subgroup B). It is a type II integral transmembrane glycoprotein in which the N-terminus of the glycoprotein is anchored via a transmembrane region and the C-terminus located extracellularly (Peter L. Collins and Mottet, 1993). The exact extent of the transmembrane domain is controversial. Collins and Mottet, (1993) suggested aa14-41 but aa20-42 has recently been suggested (Gan *et al.*, 2008). There are several forms of the SH protein present in infected cells which differ in their amount of glycosylation. The unglycosylated form (SH_0) (7.5kDa), which accumulates in abundance, a minor truncated species (SH_t) (4.5kDa) produced as the result of a second AUG translation initiation codon at methionine-23 (M23). SH_0 undergoes N-glycosylation at Asparagine-52 (N52) to form SHg (13kDa) while SHp (21-40kDa) is a post translational modification of SHg with the addition of polylactosaminoglycan to the N carbohydrates (R. A. Olmsted and Collins, 1989; Peter L. Collins and Mottet, 1993). SH accumulates in abundance in the compartments of the secretory pathway, such as the endoplasmic reticulum (ER) and the Golgi complex and associates closely with lipid raft structures within the Golgi complex (Rixon *et al.*, 2004).

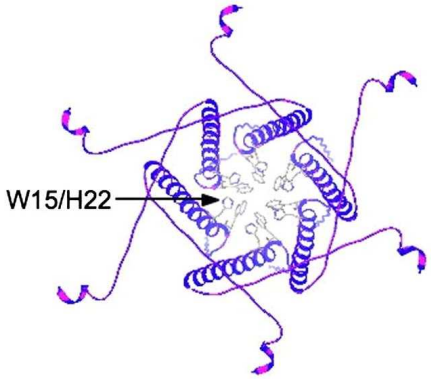

Figure 4 Molecular model of full-length SH protein as a hexamer.

W15/H22 residues within the trans-membrane domain are shown within the putative channel lumen. (Carter *et al.*, 2010).

Purified recombinant SH protein synthesized in *E. coli* were shown to form pentamers and hexamers (Figure 4) with a central channel measuring 1.9 and 2.6 nm respectively (Carter *et al.*, 2010). This model shows the location of a conserved Tryptophan-15 (W15) positioned at the channel entrance with a proximal Histidine-22 (H22). This structure is similar to that of the M2 protein of influenza virus type A which acts as an ion channel controlled by 'tryptophan gates'. Gate movement is triggered by protonation of the histidine residue (Schnell and Chou, 2008). This tryptophan gate hypotheses had been further enhanced as the transmembrane domain of the HRSV SH protein forms pentameric α-helical bundles producing cation selective ion channels in planar lipid bilayer (Gan *et al.*, 2008).

It has been suggested that the SH protein might also be involved in the enhancement of syncytium formation in cells (Heminway *et al.*, 1994). However, recombinant HRSV lacking the entire SH gene replicated as efficiently as the wild type recombinant virus in cell culture, although it grew less efficiently in the lungs of mice (A. Bukreyev *et al.*, 1997; Techaarpornkul *et al.*, 2001). Further studies of deletion mutants suggest that SH has a negative effect on virus fusion in cell culture (Techaarpornkul *et al.*, 2001). In the absence of the G gene, virus expressing both the F and SH proteins display relatively smaller plaques, reduced fusion activity and slower viral entry compared to the virus expressing the F protein alone.

The G glycoprotein

The G gene of HRSV strain A2 is 918nt in length which varies from strain to strain. The single gene encodes for two different types of glycoprotein, one being a type II integral transmembrane glycoprotein (Gm) (Satake *et al.,* 1985; Gail W. Wertz *et al.,* 1985), which is incorporated into mature virions and displayed on the infected cell surface, and a soluble form (Gs) which is secreted into the environment.

The soluble form of G glycoprotein is the result of a second in frame translation initiation codon at methionine-48 (M48) (Lichtenstein *et al.,* 1996) (see Figure 5). Gs is formed by proteolitic cleavage at the N-terminal during maturation to remove the truncated signal/anchor domain producing a polypeptide with the amino terminal asparagine residue corresponding to codon 66 (N66) of the full length G. The presence of the full length signal anchor domain prevents the cleavage of Gm suggesting the type II signal sequence (Roberts *et al.,* 1994). Hence, Gs represents the ectodomain of Gm without the signal sequence and hydrophobic transmembrane region. Gs exist as a soluble monomer (Escribano-Romero *et al.,* 2004).

Gm of strain A2 has the predicted molecular weight of 32.5kDa and the mature molecular weight of 84-90 kDa which appears as a heterogeneous band in western blots although less glycosylated bands of approximately 45-80 kDa can accumulate to a significant amount (Gail W. Wertz *et al.,* 1985). Gs is 6-9 kDa smaller compared to Gm in electrophoretic mobility (Lichtenstein *et al.,* 1996). This information suggests that more than half of the molecular weight of the G glycoprotein consists of carbohydrates especially the O-linked oligosaccharides (Gail W. Wertz *et al.,* 1985). Matured Gm oligomerizes into a homotetramer or homotrimer in the endoplasmic reticulum (Escribano-Romero *et al.,* 2004).

A new variant of subgroup B HRSV known as the Buenos Aires strain (BA) was isolated in 1999 which contains an exact repetition of 60 nucleotides in the G gene subsequent to residue 791. This repetition is flanked by a repeat of four nucleotides (GUGU) and can fold into a relatively stable secondary structure. The predicted polypeptide is extended by another 20aa (residue 260-279), reflected in the slower electrophoretic mobility of the G protein precursor of BA viruses compared with related viruses (Trento *et al.,* 2003). The antigenic significance of the repetition is currently not

known but has served as a useful natural tag in the epidemiological study of HRSV (Trento *et al.*, 2010).

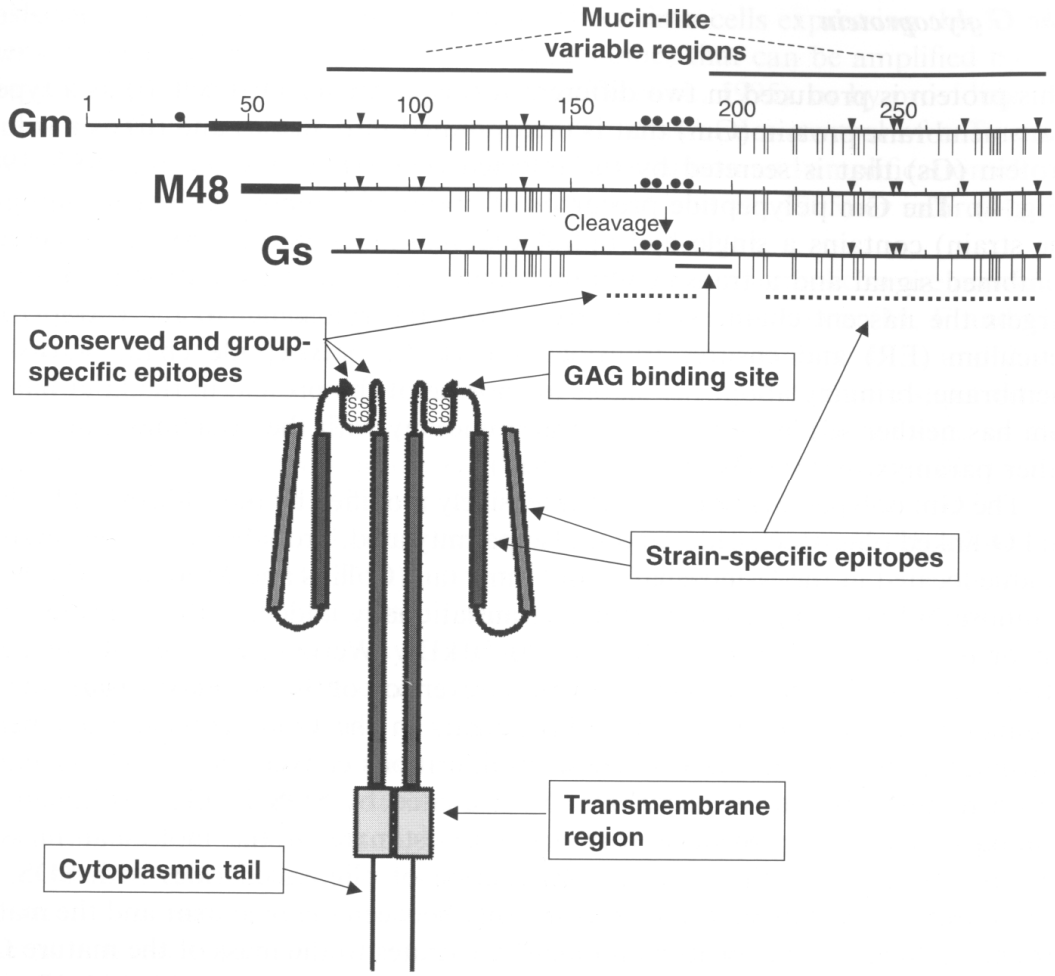

Figure 5 Schematic and 2-dimentional diagram of HRSV G glycoprotein.

A scheme of the 298 amino acid Gm protein from Long strain HRSV is presented in the upper part of the figure, denoting the hydrophobic region (▬), the potential N- (▼) and O-glycosylation sites (|), and the cysteine residues (●). Also indicated are the two mucin-like regions. Formation of the Gs form occurs by translation initiation at Met48 and subsequent cleavage after residue 65 (Roberts *et al.*, 1994). A 3D structure of the mature Gm shown as a homodimer rather than a homotetramer at the bottom of the figure for illustration purpose (Escribano-Romero *et al.*, 2004; Melero and Cane, 2006).

The first 37aa at the N-terminus form a cytoplasmic tail and is followed by the 26 hydrophobic residues of the transmembrane region. The first hypervariable region before the cysteine residues is a stiff shaft-like structure extending the polypeptide backbone towards the environment possibly due to steric interaction between the heavily clustered O-glycosylation of the region and the polypeptide (Jentoft, 1990). Both hypervariable regions are serine, threonine and proline rich which resembles the

amino acid composition of mucins as secreted by epithelial cells, and are named mucin-like regions (I. Martinez *et al.*, 1997).

The epitopes of the G glycoproteins were grouped into three regions namely the conserved, subgroup specific and strain specific epitopes based on the sequence analysis of escape mutants selected against a panel of monoclonal antibodies. Briefly, virus plaques were passaged in the presence of monoclonal antibodies in the form of hybridoma supernatant which were raised against a subgroup A HRSV strain Mon/3/88 and the G gene of the resulting escape mutants were sequenced and analysed (I. Martinez *et al.*, 1997). Other methods such as competitive ELISA have been used for topological mapping of epitopes in the G glycoprotein and ten distinct epitope regions were identified based on the panel of monoclonal antibodies used (B Garcia-Barreno *et al.*, 1989).

The central unglycosylated region contains both conserved epitopes for all HRSV and subgroup specific epitope which are unique among HRSV from the same subgroup. The central unglycosylated region contains a 13aa sequence which is highly conserved among all HRSV strains and the 'cysteine noose' region overlaps four cysteine residues (aa 173, 176, 182, 186), also conserved in BRSV, which forms disulphide bonds between C173-C186 and C176-C182 holding this region like a hairpin loop (I. Martinez *et al.*, 1997; Melero *et al.*, 1997). The cysteine noose starts with a single α-helix followed by a 1.5 α-helical turn formed by residue P180-W185 in which the inner disulphide bridge (aa176-182) adopts the left hand conformation but the outer disulphide bridge (aa173-186) is less defined. The outer disulphide bridge probably adopts both left and right hand conformation which exist in equilibrium. The two α-helices are connected by a reverse type I turn (aa Cys176-Asn179) and form a 9Å thick disk with a diameter of approximately 20Å with Asn179 extending into the environment. A hydrophobic pouch of approximately 4Å is thought to attach to small ligands or amino acid side chains. The hydrophobic pouch is lined by Cys176-Cys182 (top), Ile185-Cys186 (right), Val171 and Pro172 (bottom) and Cys173 (left). The Cys182 has the most surface accessibility with the backbone oxygen atom being the only exposed hydrophilic atom in the pouch and is hydrogen bonded with the amide hydrogen of Cys186 (Doreleijers *et al.*, 1996).

The C-terminal hypervariable region of the Gm after the cystine noose contains strain specific epitopes (I. Martinez *et al.*, 1997). This region is prone to host protease

degradation and viruses grown in Vero cells exhibit a mature G protein which lacks the C-terminus and has a molecular weight of 55 kDa as compared to 80-90 kDa in HRSV grown in Hep-2 cells (Kwilas *et al.*, 2009). The C-terminal hypervariable region is also highly O-glycosylated as with the first hyperviariable region and host cell specific glycosylation affects the expression of some strain specific epitope (Garcia-Beato and Melero, 2000). Thus, infection of a HRSV strain in different individuals might produce G glycoprotein with different carbohydrate side chain due to individual polymorphisms which alter the expression of epitopes in the G glycoprotein (Melero *et al.*, 1997). The glycosylation of the C terminal hypervariable region seems to reduce the reactivity to human sera suggesting that glycosylation in this region is important as part of the viral immune evasion strategy (Rawling and Melero, 2007).

The G glycoprotein was shown to play a role in attachment of HRSV. The use of G specific monoclonal antibodies blocks the attachment of wild type (wt) HRSV to HeLa monolayer (S. Levine *et al.*, 1987). However, G gene deletion mutant viruses remained capable of infecting cells but with reduced efficiency of entry by 3 to 4-fold (Schlender *et al.*, 2003).

The G glycoprotein contains a heparin binding domain spanning from aa 184-198 in subgroup A and aa 183-197 in subgroup B (Feldman *et al.*, 1999). Treatment of HRSV with heparin blocks *in vitro* infection of Hep-2 cells monolayer but only with the presence of heparin during the time of inoculation. Further experimentation with immobilized heparin and affinity chromatography confirms the binding of G to heparin (Krusat and Streckert, 1997). The G glycoprotein also binds to heparin-like glycosaminoglycans (GAGs) such as heparan sulphate expressed on the cell surface (Isidoro Martinez and Melero, 2000) which is also abundant in the lung.

The Gs is produced in much larger quantity compared to the membrane bound G (Gm) (Roberts *et al.*, 1994) in the first 24 hours p.i. and comprises 20% of the total G glycoprotein produced during infection. Gs was shown to play a role as an antigen decoy to facilitate immune evasion. Although the initial amount of Gs in the inoculum is insufficient to protect the virus from G specific antibodies from maternal transfer or previous infections, the local concentration of Gs surrounding the infected epithelial cell would be significant after one or two cycles in replication and may be enough to quench G specific antibodies (Alexander Bukreyev *et al.*, 2008).

The central conserved cysteine noose contains a CX3C chemokine motif at amino acid position 182-CWAIC-186 which binds to CX3CR1, a specific receptor for the CX3C chemokine fractalkine (CX3CL1) which regulates leukocyte adhesion, activation, and trafficking (Tripp *et al.*, 2001). This finding suggests that the CX3C motif is involved in the induction of pathology in RSV infected tissues *in vivo.* The CX3C motif was proven to be important in the development of enhanced disease caused by FI-RSV. Mice immunized with FI-RSV vaccine with an ablated CX3C motif or the deletion of the whole G did not exhibit enhanced pulmonary disease characterised by pulmonary eosinophilia with significant inflammatory response (Haynes *et al.*, 2003). Immunization of cotton rats with recombinant HRSV without the G glycoprotein (GΔHRSV) induced long lasting protection with the antibody titer comparable with cotton rats immunized with wtHRSV. The GΔHRSV detectability in the nose and lungs of cotton rats was negligible compared to wtHRSV but low levels could be detected in nasal washes showing GΔHRSV is highly attenuated *in vivo* but indistinguishable *in vitro* (Widjojoatmodjo *et al.*, 2010).

The Fusion glycoprotein

The F gene of HRSV strain A2 is 1903 nucleotides in length and contains a single ORF which encodes a protein of 574 aa with the calculated molecular weight of 63.5kDa (P. L. Collins *et al.*, 1984b). Unlike the SH and G protein, the fusion (F) glycoprotein is a type I integral membrane protein whereby its N-terminus is oriented extracellularly while the C-terminus remains in the cytoplasm (P. L. Collins *et al.*, 1984b). There are three hydrophobic regions in the F glycoprotein as shown in Figure 6. First, the signal sequence in the N terminus of the F2 chain, second is the fusion peptide at the N-terminus of the N-terminus of F1 chain, and the third is the transmembrane regions located near the C-terminus of F1 chain. Adjacent to the fusion peptide and transmembrane regions are two heptad sequences namely, HRA and HRB. HRA and HRB peptides are known to form trimeric complexes in solution (Matthews *et al.*, 2000; Xun Zhao *et al.*, 2000).

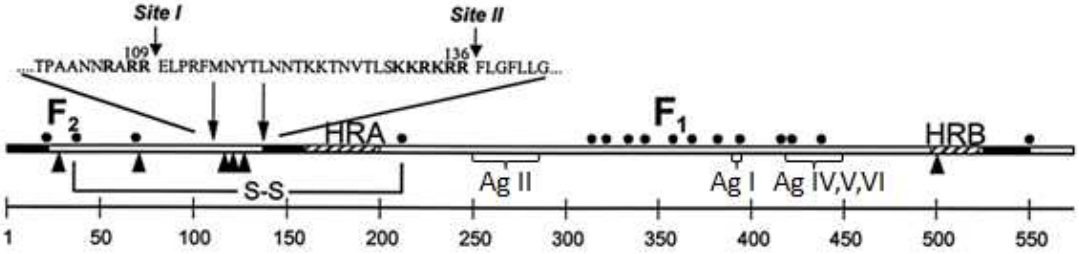

Figure 6 Schematic diagram of the F glycoprotein.

Diagram of the F protein primary structure denoting cysteine residues (●), potential glycosylation sites (▲), hybrophobic regions (■), heptad repeat sequences (▨) and cleavage sites by fur-sequence motifs (boldface) the cleavage sites I and II (Gonzalez-Reyes *et al.*, 2001) and the antigenic sites (Ag) I, II, IV,V,VI) are indicated (Lopez *et al.*, 1998).

F is initially synthesized as an inactive precursor (F0) and translocated through the membrane into the lumen of the endoplasmic reticulum initiated by the signal sequence at aa1-22 where high mannose sugar chains are covalently added to the asparagine residues. There are a total of 5 and 6 potential N-glycan acceptor residues in the F0 (A2 and Long respectively). Then F is exported to the distal cisternae of the trans Golgi compartment and the more distal trans Golgi network where the furin like protease will recognise the highly conserved basic motifs, 106-RARR-109 (*Site I*) and 131-KKRKRR-136 (*Site II*) and cleaved at both residues 109 and 136 (Figure 6) resulting in two phenomenon; first, the release of a 27 aa peptide (pep27) the exact function of

which is not yet determined, second, the formation of polypeptides (F2) from the N-terminus and F1 from the C terminus which are held together by a disulphide bond. The mature F protein can be resolved under reducing condition in SDS-PAGE as bands of 50 kDa (F1), 20 kDa (F2) and 58 kDa (cleavage intermediates) (Zimmer *et al.*, 2001). The cleavage intermediates resulting from inefficient protease activity can also be found in HRSV infected cells (Gonzalez-Reyes *et al.*, 2001). No F0 residue is found on the cell surface and export of F to the cell surface solely depends on cleavage and not glycosylation (Peter L. Collins and Mottet, 1991).

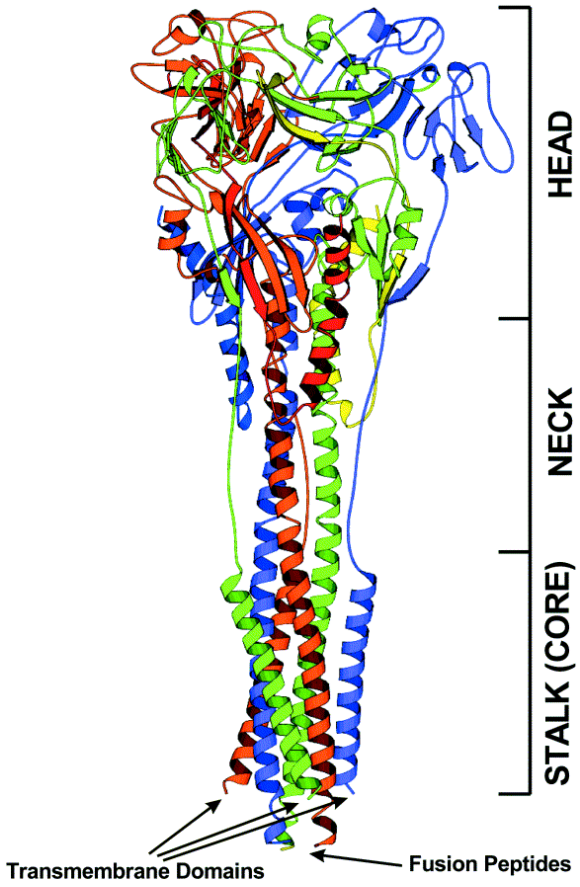

Figure 7 Homotrimeric fusion glycoprotein.

Diagrammatic representation of the pre-fusion structure of the homology model of RSV-F. The protein is colored by chain, with the F_1 subunits in orange, green, and violet and the F_2 subunits in red, yellow, and blue. The division of the structure into head, neck, and stalk (or core) regions is indicated, as are the attachment points for the three viral anchor peptides and three fusion peptides, which are not included in the model. (Morton *et al.*, 2003).

The prefusion mature F glycoprotein folds into a homotrimer (Calder *et al.*, 2000) as depicted in Figure 7 before being exported onto the cell membrane with the transmembrane anchor residues 525-550 embedded in the lipid bilayer (P. L. Collins *et*

al., 1984b) the fusion peptide protruding from the N-terminus of the F2 (Matthews *et al.*, 2000).

The trimeric structure can be divided into three segments namely the stalk/core, neck and head. The core is made up by a six-membered antiparallel helical coiled coil structure where the HRA and HRB interacts (Xun Zhao *et al.*, 2000).

The head region of each monomer is made up by two β-coiled domains, namely I and II which form the upper walls of a radial channel at intersection of the head and the neck layers. Domain I is a highly twisted 7-stranded β barrel-like assembly and domain II is an immunoglobulin-like sandwich domain and it forms the opposite wall of the radial channel. A central void accessible from the exterior of the protein through a channel down the axis of the protein can be observed by electron microscopy (Lin Chen *et al.*, 2001). The head is supported by the protruding neck region (Morton *et al.*, 2003) made up of a triple-helical coiled coil encircling three copies of residue G171-F221 and extended to the HRA region by 21 residues (Lin Chen *et al.*, 2001). The HRA coiled coil continues towards the N terminus, out of the base of the neck and onwards to the base of the stalk where the remaining visible HRA region is well packed around the molecular axis (Lin Chen *et al.*, 2001).

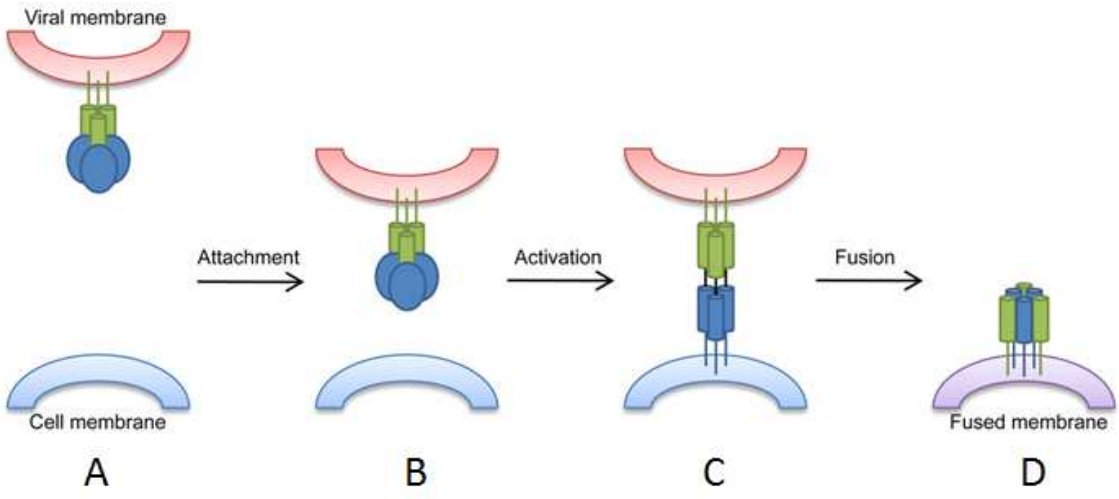

Figure 8 Membrane fusion mechanisms (McLellan *et al.*, 2010)

The fusion mechanisms of the F glycoprotein are depicted in Figure 8 . In the prefusion state, the fusion peptide is always kept at the end of HRA (Figure 8A and B) and upon attachment to a cell, the fusion protein is activated and forms a prehairpin intermediate resulted in the release of the fusion peptide through the head of the fusion protein into the target cell membrane (Figure 8C). The target cell membrane and viral membrane are pulled into close proximity when the prehairpin intermediate structure collapses into the hairpin intermediate and the six helix bundles begins to form resulting in the formation of post fusion conformation where the membrane fuses together (Figure 8D) (Morton *et al.*, 2003; McLellan *et al.*, 2010).

The initial work to map the epitopes of the fusion glycoproteins revealed three distinct types of epitopes namely; neutralizing and fusion inhibiting, neutralizing, and non-neutralizing (Edward E. Walsh *et al.*, 1986). Later, these antigenic sites were mapped into domains called I, II, IV, V, and VI (('Recovery from infants with respiratory illness of a virus related to chimpanzee coryza agent (CCA). II. Epidemiologic aspects of infection in infants and young children.,' ; Arbiza *et al.*, 1992) and the locations are shown in Figure 6.

Antigenic site II is located in the protease resistant region (aa255-275) within the F1 (Lopez *et al.*, 1998) and antibodies that bind to antigenic site II are highly neutralizing and possess protective fusion inhibitory property ('Recovery from infants with respiratory illness of a virus related to chimpanzee coryza agent (CCA). II. Epidemiologic aspects of infection in infants and young children.,' ; C. E. Taylor *et al.*, 1989). Antigenic site II is made up by two α-helices which require a certain level of conformational protein folding for the correct presention of the functional epitope, thus mapping antigenic site II with shorter overlapping peptides tend to give negative results (Lopez *et al.*, 1993). The humanized murine monoclonal antibody (Palivizumab) which is currently used as a passive immune prophylaxis against HRSV infection (Melero and Cane, 2006) binds to this site.

Antigenic site IV, V and VI are neutralizing epitopes and binding of recombinant murine monoclonal ch101F which was mapped to aa 422-438 within this site neutralized the wild type HRSV (Sheng-Jiun Wu *et al.*, 2007b). Site I is a group A specific epitope which is partially neutralizing with one log unit reduction in infectivity with Long strain but escape mutants to this site can be recovered after passaging the virus under selective pressure with monoclonal antibodies to this site. Escape mutants

derived from Mabs to this site revealed at least two distinct epitopes. (Lopez *et al.*, 1998).

1.5 **Epidemiology**

RSV epidemics occur each winter in temperate climates such as in the UK starting around week 40 reaching a peak in between week 48-52 as shown in Figure 9. During the peak of the epidemic, there can be more than 1000 cases of HRSV infection reported every week in the UK with a majority of the patients aged <1 yr followed by those of 1-4 yrs (Patricia. A. Cane, 2001). HRSV is also recognized as a major respiratory pathogen in some adult populations (A.R. Falsey *et al.*, 2005) especially those with cardiopulmonary diseases, and among the immunocompromised hosts such as those with human immunodeficiency virus (Dianne Murphy and Rose, 1989; van der Ven *et al.*, 1996), renal transplant patient(s) (Janet A. Englund *et al.*, 1988; Morales *et al.*, 2003) and pancreas transplant patients (Janet A. Englund *et al.*, 1988). The epidemiologic study revealed that HRSV is responsible in 2-5% of pneumonia in the adult population. The HRSV attack rate in the nursing home was estimated at 5-10% annually with 10-20% HRSV associated pneumonia and 2-5% mortality (Ann R. Falsey and Walsh, 2000). Patients scheduled for bone marrow transplant are at a very high risk due to the administration of immunosuppresive drugs prior to marrow engraftment (Ann R. Falsey and Walsh, 2000) although early administration of ribavirin might prevent the progression of URTI to LRTI and reduced mortality rate in those who developed pneumonia (Hattington *et al.*, 1992; Bowden, 1997).

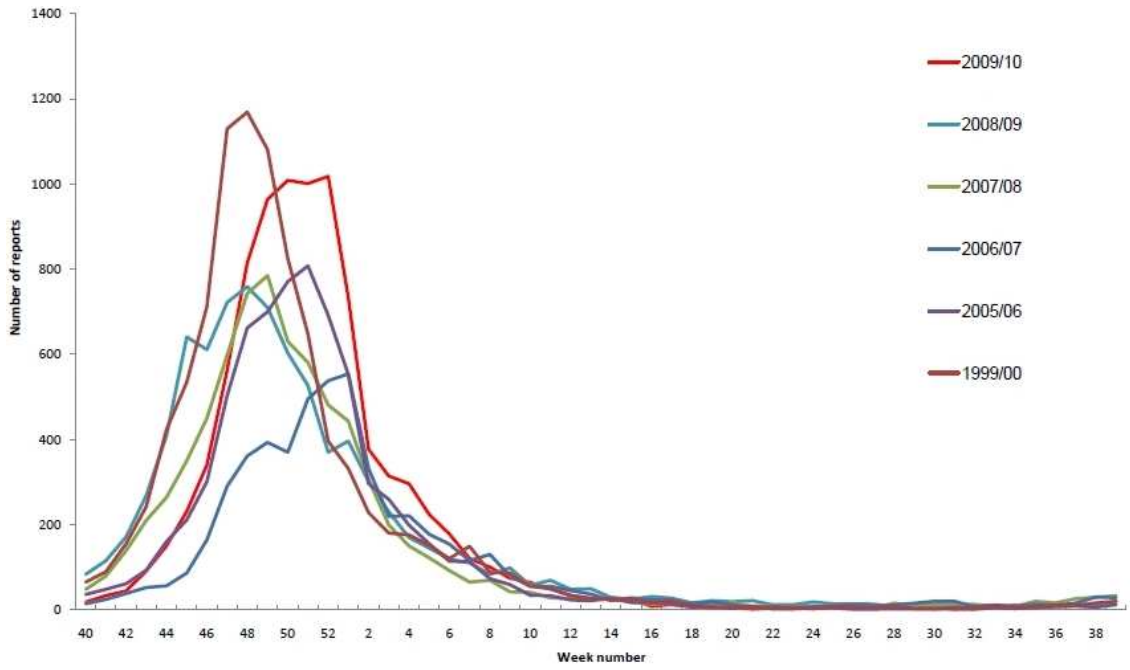

Figure 9 Prevalence of HRSV infection in the UK.

The graph was adapted from http://www.hpa.org.uk/ updated on the 1st September 2010 as reported by the microbiology laboratories of NHS and HPA over 6 seasons based on the date of specimen.

In equatorial countries such as Malaysia, HRSV is endemic throughout the year with a periodic peak between November to January annually (see Figure 10) corresponding to the annual monsoon season which is characterized by a dramatic increase in rainfall (Chan *et al.*, 2002). However, it is noteworthy that the percentage of cases of HRSV reported in the drier months may not be significantly lower than those reported during the monsoon season. The epidemics seem to emerge in the equatorial regions and radiates in both direction north to the northern hemisphere and south to the southern hemisphere (Stensballe *et al.*, 2003).

The compilation of published epidemiological data revealed a distinct epidemic pattern according to region with countries from each region sharing similar seasonality and epidemic peak. In every region, the annual temporal peak occurs in a relatively fixed epicenter in the same month with spreads over the following months away from the epicenter corresponding to the gradual climatic change. Nevertheless, all epidemic peaks are associated with factors such as population crowding, reduced herd immunity especially among child bearing females which leads to infants with reduced immunity and seasonal immunosuppression (Stensballe *et al.*, 2003).

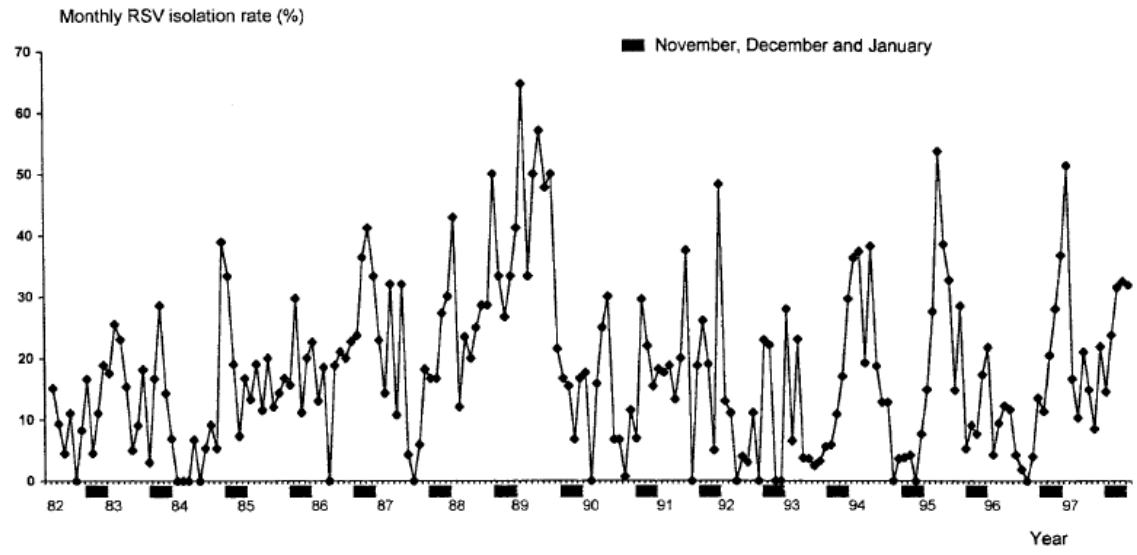

Figure 10 Prevalence of HRSV infection in Malaysia for 180 months between January 1982 to December 1997

Only patients with LRTI were included (n=5691, age<24 months).

1.5.1 **RSV subgroups**

HRSV isolates can be categorized into 2 subgroups identified as subgroups A and B. They are effectively 2 different serotypes which can be distinguished with polyclonal antibodies in plaque reduction neutralization assays where polyclonal raised against one subgroup can effectively neutralize its homologous subgroup but is less effective against the heterologous subgroup (Coates *et al.*, 1966). Later, the subgrouping was standardized by the reactivity profile with a panel of monoclonal antibodies directed against the P, F and G proteins by IFA or ELISA (Anderson *et al.*, 1985), G, F, M, N by radioimmunoprecipitation analysis and IFA (Mufson *et al.*, 1985), F and P by IFA and immunoblotting (H. B. Gimenez *et al.,* 1986) F, G, N and P by IFA (Tsutsumi *et al.*, 1988).

During an epidemic, both subgroups of HRSV are normally present with varying proportion with either one subgroup dominating or in equal proportions (Mufson *et al.*, 1988; Hendry *et al.*, 1989; Hall *et al.*, 1990; Freymuth *et al.*, 1991). Attempts to measure and compare the severity of disease caused by both group of viruses have not yielded a clear cut differences in virulence (G. McIntosh *et al.*, 1993; Martinello *et al.*, 2002; Oliveira *et al.*, 2008). Nevertheless, it was tempting to speculate that subgroup A causes more severe disease compared to subgroup B (Hall *et al.*, 1990; Edward E. Walsh *et al.*, 1997; Papadopoulos *et al.*, 2004).

The antigenic relatedness between sugroup A and B was estimated from antibody response by cotton rats immunized with either subgroup A or subgroup B HRSV. It was shown that the fusion glycoprotein shares 50% relatedness while the G glycoprotein shares only 5% relatedness in murine model. Both subgroups shared only 53-56% amino acid identity in G between subgroups A (strain A2) and B (strains 18537 and 8/60) (Wayne M. Sullender *et al.*, 1990). The authors suggested that the fusion glycoprotein is responsible for most of the observed cross subgroup neutralization and protection in the murine model (Anderson *et al.*, 1985; P. R. Johnson, Jr. *et al.*, 1987). Their work was further confirmed in the human model by Hendry et al.,(1988) where serum from infants infected with HRSV responded to the fusion protein of both homologous and heterologous strains of HRSV without any significant difference. However, the authors reported a significant difference when assayed on the G glycoprotein with only 7.3% relatedness between subgroups A and B. Nevertheless, the similarities in the amino acid sequence can be summarised as in Table 1. The authors

did not state the HRSV strains used for comparison and the interpretation of this table should be done with care.

Table 1 Percentage (%) amino acid sequence identity for proteins in HRSV

	NS1	NS2	N	P	M	SH	G	F	M2-1	M2-2	L
% identity	87	92	96	91	91	76	53	89	92	72	93

Data was adapted from Collin and Crowe (2001)

Molecular evolutionary studies on the G glycoprotein gene of subgroups A and B revealed a very similar evolutionary rate of 1.83×10^{-3} nucleotide substitutions/site/year (Zlateva *et al.,* 2004) and 1.95×10^{-3} nucleotide substitutions/site/year (Zlateva *et al.*, 2005) respectively. However, the analysis of the G gene of the BA viruses alone revealed a higher evolutionary rate of 4.7×10^{-3} nucleotide substitutions/site/year (Trento *et al.,* 2010), more than double the evolutionary rate of the regular subgroup B viruses. The evolutionary rate for the fusion glycoprotein was estimated to be 1.3-1.5 x 10^{-3} nucleotide substitutions/site/year showing a higher selective pressure against the G glycoprotein than F glycoprotein suggesting the immunologic pressure exerted on the G glycoprotein is the main factor behind the diversification of HRSV lineages (Gaunt *et al.,* 2011).

Probabilistic models of codon substitution that allow for variable nonsynonymous/synonymous substitution rate ratios among sites revealed that both subgroups A and B differ in positively immune-selected codons in the G glycoprotein (Zlateva *et al.,* 2004; Zlateva *et al.,* 2005) except codon 226 in subgroup A which corresponds to codon 227 in subgroup B (Woelk and Holmes, 2001) suggesting a different selective pressure between subgroups and probably utilizing a different host immune evasion strategy.

1.5.2 **Genotypes**

The variation between isolates from the same subgroup was initially demonstrated with monoclonal antibodies raised against the prototype strain (Patricia. A. Cane, 2001). This was followed by other methods and the most commonly used being the phylogenetic analysis of the G nucleotide sequence which can accurately define the number of genotypes within a subgroup (Peret *et al.*, 2000; Trento *et al.*, 2006; Trento *et al.*, 2010).

In any one epidemic, more than one genotype from each subgroup may circulate (Lim *et al.*, 2003). One genotype may be dominant in one epidemic but forms a minority genotype in the following epidemic and may disappear altogether in the third epidemic, perhaps replaced by a new genotype (Peret *et al.*, 2000; Patricia. A. Cane, 2001).

Clinical severity between the genotypes of subgroup A HRSV has been evaluated and genotype GA3 seems to produce more severe disease compared to GA2 and GA4 (Martinello *et al.*, 2002) but recent reports showed no correlation between severity of disease with genotype (Rong-Fang Zhang *et al.*, 2010a) although the genotypes evaluated were not the same with the previous. Comparing disease severity between genogroup might not be entirely possible due to the shift of the predominant group in each epidemic and the existence of heterogenous genotypes across the globe.

1.6 Infection, Pathogenesis and Immunity

1.6.1 Spread

HRSV can be considered as the one of the highly prevalent viruses that affects humans and is mainly spread through self inoculation by contact with surfaces contaminated by RSV-infected nasal secretions (Hall *et al.*, 1980). HRSV is more labile when compared to rhinovirus but survived long enough outside host environment for transmission. In an experiment done back in 1980 shows that HRSV from nasal secretions remains infectious on countertops up to 6 hours and on hands up to 25 minutes (Hall *et al.*, 1980). The spread through aerosols seems unlikely as the virus was quickly inactivated upon drying (Rechsteiner and Winkler, 1969) and infectious particle transmission are often through large droplets (Hall *et al.*, 1978). Infection can be efficiently established by self inoculation through the eyes or nose but not through the mouth (Hall *et al.*, 1981).

1.6.2 Infection

Virus replication starts in the nasopharynx with the incubation time around 3-5 days (Sterner *et al.*, 1966) with the highest detectable virus titer at day 6-8 p.i (Kim *et al.*, 1971) with the peak illness occurred around day 8-10 post infection (Sterner *et al.*, 1966; Wright *et al.*, 2002).

HRSV only causes upper respiratory tract (URT) infection in most infants ranging from mild medical manifestation such as poor feeding, rhinorrhea, coughing, fever, apnea, lethargy wheezing, respiratory distress (Baker and Ryan, 1999), and otitis media (T. Heikkinen *et al.*, 1995; Terho Heikkinen *et al.*, 1999). However, in some HRSV naïve infants, infection can quickly spread to the lower respiratory tract (LRT) resulting in bronchiolitis and pneumonia, and such cases are responsible for about 1-2% infant hospitalizations of which 2-5% required mechanical ventilation (Glezen *et al.*, 1986; C. L. Collins and Pollard, 2002).

The clinical manifestation of HRSV infection correlates with the pathology caused by the virus in the LRT. In acute bronchiolitis, the main pathology is epithelial necrosis of the small airways. The bronchiolar lumen may be blocked by dense mucus plugs leading to trapping of air and mechanical interference with ventilation. In pneumonia,

there is a widespread inflammation and necrosis of the lung parenchyma, and severe lesions of the bronchial and bronchiolar mucosa (Aherne *et al.*, 1970).

Clinical recovery usually corresponded to the termination of virus shedding although some infants continue to shed virus for many weeks after recovery (Wright *et al.*, 2002).

HRSV only causes localized respiratory infection in normal infants and adults but systemic infection and spread to other organs such as liver, heart and kidney in immune-compromised individual had been documented (M Fishaut *et al.*, 1980; Milner *et al.*, 1985; Padman *et al.*, 1985).

1.6.3 **Innate immunity**

The first line of innate defence mechanism is associated with the surfactant protein (SP), a member of collectin family in the respiratory system. It was shown in infants that the amount of surfactant protein present the bronchioaveolar lavage fluid in children with severe HRSV associated LRTI shows a marked reduction in concentration suggesting the importance of surfactant protein in keeping infecting HRSV at bay (Kerr and Paton, 1999; Barreira *et al.*, 2010; Kawasaki *et al.*, 2011). Later, HRSV infection of the pulmonary bronchiolar epithelial cells shows an inhibitory effect to the translation of SP-A mRNA although SP-A mRNA transcription was not impaired (Bruce *et al.*, 2009). *In vitro* experimentation has shown a virus neutralizing effect of SP-A which recognizes the fusion glycoprotein rather than G glycoprotein (Ghildyal *et al.*, 1999). *In vivo* studies with SP-A deficient mice (Ann Marie LeVine *et al.*, 1999) and SP-D deficient mice (Ann Marie LeVine *et al.*, 2004) showed the importance of SP-A and SP-D in the enhancement of virus clearance.

When infection sets in the airway epithelial cells of mice, these cells together with dendritic cells (DC) and macrophages are the first cells to respond to HRSV infections (Sung *et al.*, 2006). The presence of different DC subsets will determine the course of the immune response. For example, the expansion of plasmacytoid DC (pDC) and conventional DC (cDC) was shown to have a protective impact to the airway by reduced pathophysiology characterized by decreased airway hypersensitivity, reduced Th2 cytokines, increased Th1 cytokines and a reduction in airway inflamation and mucus overexpression. All these protective responses can be reversed by the selective depletion of pDC (Smit *et al.*, 2008) which resulted in decreased HRSV clearance and leads to the

manifestation of immune-mediated pathology caused by HRSV infection in BALB/c mice (Smit *et al.*, 2006).

HRSV infection is detected thorugh a series of pattern recognition receptors (PRR) comprising of Toll-like receptors (TLR) and cytoplasmic sensors of viral RNA, such as retinoic-acid inducible protein I (RIG-1) and melanoma-differentiation associated gene 5 (MDA-5) in mice (Kato *et al.*, 2006). The initial detection of HRSV in the airway epithelial cells is recognised by RIG-1 receptors due to the presence of single stranded virus RNA (Ping Liu *et al.*, 2007) which contains uncapped unmodified 5'-triphosphate RNA (Pichlmair *et al.*, 2006). The synthesis of double stranded RNA later in HRSV replication process stimulates the TLR3 (Ping Liu *et al.*, 2007). Upon stimulation , RIG-I and TLR3 activates NF-kappaB through a MyD88 (adaptor protein shared by all TLR) dependent pathway, activates mitogen-activated proteins (MAP) kinases independent of MyD88 pathway, which stimulates the production of type I interferons, (INFs) and promotes the maturation of DCs (Alexopoulou *et al.*, 2001). Type I INF like INF-α, INF-β and INF-ω are key cytokines to antiviral response (Muller *et al.*, 1994), induce apoptosis in murine fibroblast cells, limits virus replication in infected cells and prevents the spread of infection to non infected cells (Balachandran *et al.*, 2000).

The F glycoprotein of RSV was shown to stimulate the synthesis of interleukin 6 (IL-6) by purified human monocytes expressing TLR4, essential receptor for the recognition of lipopolysaccharides from Gram negative bacteria, mycoplasmas, spirochetes and fungi. The G glycoprotein and nucleocapsid protein stimulates weaker cytokine response than F (Kurt-Jones *et al.*, 2000). The interation between the F glycoprotein and TLR4 is host species specific which is not activated upon stimulation by BRSV derived F glycoprotein (Lizundia *et al.*, 2008). The deficiency of TLR4 results in prolonged infection (Kurt-Jones *et al.*, 2000) in human and reduced NF-kappaB activity in mice (Haeberle *et al.*, 2002). Genetic polymorphism studies have shown mutation in autosomal gene encoding TLR4 being associated with severe HRSV disease in human (Mailaparambil *et al.*, 2008). HRSV infection in the airway epithelial cells induces the numerous cytokine upregulation genes resulting in the accumation of an array of cytokines such as CC [RANTES, Macrophage chemoattractant protein (MCP)-1α, -1β], CXC [inducible neutrophil chemotactic factor (GRO)-α, -β, -γ and IL-8) and CX3C (fractalkines) which promotes the recruitment of neutrophils, monocytes, DC and T cells into the respiratory tract, possibly contributes to a more severe disease (Sheeran *et*

al., 1999; Allison *et al.*, 2004). High levels of TNF-α, RANTES, MIP-1α, IL-6, IL-8, and IL-10 were detected in the nasal wash and tracheal aspirates of patients with severe HRSV infection (Olszewska-Pazdrak *et al.*, 1998; Sheeran *et al.*, 1999; Gill *et al.*, 2008). However, single nucleotide polymorphisms (SNP) studies have revealed the association of SNPs in the gene loci of IL-4 (Forton *et al.*, 2009), IL-8 (Hacking *et al.*, 2004; Lu *et al.*, 2010), IL-9 (Schuurhof *et al.*, 2010), IL10 (Wilson *et al.*, 2005) and TNF-α (Puthothu *et al.*, 2009) as a genetic determinants of severe HRSV disease in infants.

1.6.4 **Adaptive immunity**

The stimulation of the adaptive immunity by HRSV is incomplete and reinfections is common and occurs throughout life (Hall *et al.*, 1991). After HRSV infection is established, cDC migrates to the lymph nodes presenting the HRSV derived antigens to the naïve T and memory specific T cells. Some migratory DC might have been infected by HRSV but HRSV antigens derived from infected epithelial cells might have contributed to the antigen display to the T cells (Lukens *et al.*, 2009) as human DC infected with HRSV was shown to be a poor inducer for T-cell proliferation (de Graaff *et al.*, 2005).

Two novel CD4+ T cell epitopes had been identified to date in the M and M2 protein corresponding to the sequence 213-FKYIKPQSQFI-223 and 27-YFEWPPHALLV-37 respectively. Peptides containing these epitopes can stimulate the proliferation of mainly lung CD4+ T cells in greater ratio compared to the spleen CD4+ T cells to produce INF-γ, IL-2 (Jie Liu *et al.*, 2009) and IL-10 (F. Eun-Hyung Lee *et al.*, 2005) in murine models. CD4+ T cells were found to mediate the synthesis of Th2 related chemokines and the depletion of CD4+ T cells induced reduced airway hyperresponsiveness (AHR), lower Th2 cytokines and reduced Gob5 production in mice model (Neupane *et al.*, 2007). CD4+ T cells associated cytokines promotes the recruitment and infiltration of neutrophils and eosinophils into the lung tissues (Bueno *et al.*, 2008). Following this INF-γ promotes cytotoxic T cells function by stimutating CD8+ T cells and Natural Killer (NK) cells to clear virus infected cells, stimulate macrophage phagocytic activity to eliminate dead cells and induce the production of IgG antibodies by B cells (Kidd, 2003). However, when immune response to HRSV infection is skewed towards the Th2 cells mediated pathways, IL4 and IL5 was secreted by innate immune cells into the blood and directs strong pulmonaric eosinophil

response as observed in FI-RSV enhanced disease in murine models (P. J. M. Openshaw *et al.*, 2001; Becker, 2006) leading to enhanced pathology to the lungs.

Meanwhile, CD8+ T cells undergo rapid expansion and 50-70% were virus specific as estimated with LCMV infection in mice (Murali-Krishna *et al.*, 1998). CD8+ cytotoxic lymphocytes (CTLs) activity peaks around day 6 post immunization by predominantly recombinant M2 protein in mice with residual activity detected 45 days post immunization showing the resistance induced by M2 protein was mainly mediated by primary pulmonary CTLs supporting the observation of frequent HRSV reinfection in humans (Kulkarni *et al.*, 1993). Depletion of CD8+ CTLs in HRSV challenged mice showed elevated AHR, production of IL-13 and increased mucus associated gene (Gob5) expression (Neupane *et al.*, 2007). In sufficient numbers, CD8+ CTLs inhibits Th2 associated chemokines and prevents the progression of eosinophilia BALB/c mice (Olson *et al.*, 2008) and are not associated with severe HRSV disease (Lukens *et al.*, 2010). However, the contribution of neutrophils influx into the lung tissues due to Th1 mediated cytokines to the lung pathology have not been established in mice (Lukens *et al.*, 2010).

Thus, it is clear that severe HRSV disease is not solely caused by virus mediated pathology but a great proportion was driven by immune response by the Th2>Th1 skewed adaptive immunity. Other than that, SNPs to key genes in the innate immune systems might predispose individuals to genetic mediated risk during HRSV pathogenesis. Nevertheless, most experiments had been done in mouse model which is not a natural host for HRSV and may represent a totally different picture in human humoral response.

Infants acquire maternal IgG starting from week 26 to birth. Although some infants do develop severe HRSV disease, maternal antibodies seem to offer some protection and are associated with milder disease outcome in infants (Glezen *et al.*, 1981a). Premature infants tend to be at a higher risk of acquiring severe disease, and full term infants tend to be spared to a certain extent in the first month of life (P. L. Collins and Crowe, 2001). The sensitivity of very young infants in developing infectious disease such as HRSV infection might be due to the lack of preexisting immunological memory and delayed neonatal B cell responses (Adkins *et al.*, 2004). Healthy institutionalised elderly have intact humoral response and are not associated with severe HRSV disease (Ann R.

Falsey and Walsh, 1992) but frail elderly have waned HRSV antibody which might exposed them to a higher risk of HRSV reinfection (Terrosi, 2009).

The humoral response in infants 2 months and below is mainly restricted to IgA response but is not neutralizing (Wright *et al.*, 2000). However the initial appearance of IgA antibody to HRSV marked the termination of an infection and continued to rise thereafter (Kenneth McIntosh *et al.*, 1978). The detection of HRSV specific IgE in HRSV infants during bronchiolitis have been associated with wheezing and asthma later in life (Welliver and Duffy, 1993) but IgE was not detected in a separate groups of infants (Rakes *et al.*, 1999; de Alarcon *et al.*, 2001) suggesting the production of IgE, a product of Th2 lymphocytes response is not mandatory in all HRSV infections.

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1.7 Vaccines

1.7.1 Formalin inactivated RSV (FI-RSV)

This method involved the formalin inactivation of Bernett strain of RSV grown from vervet monkey kidney cell followed by concentration by centrifugation and absorption into adjuvant aluminium hydroxide. FI-RSV lot 100, Pfizer was administered intramuscularly (Delgado *et al.*, 2009) in three injections to children aged 4 months to 7 years prior to the annual RSV epidemic. However, the increase in neutralizing antibody of the vaccinees to HRSV infections was not promising (47%) and failed to protect the vaccinees from the natural infection of environmental RSV. However, all vaccinees shown at least four-fold increased in serum complement fixation (CF) antibody (Fulginiti *et al.,* 1969). To make things worse, RSV-naïve vaccinees experienced respiratory disease enhancement when exposed to the natural infection in the epidemic post-vaccination (Chin *et al.*, 1969). No children previously exposed to wild type HRSV infection developed disease enhancement after acquiring HRSV infection post vaccination (Fulginiti *et al.*, 1969). None of those who experienced with disease enhancement acquired a second disease enhancement in the following epidemics. This was probably due to the B cells elicited by wtHRSV outcompeting the pathogenic B cells primed by FI-RSV and establishing a normal response to future reinfections (Delgado *et al.*, 2009). Enhanced disease was also reported in cynomolgus macaques vaccinated with FI human metapneumovirus (FI-HMPV) showing the similar drawback consequences as FI-RSV (de Swart *et al.*, 2007).

The alteration to the protective epitopes by formalin was thought to be the main reason in the lack of neutralizing antibodies in HRSV naïve vaccinees (Brian R. Murphy *et al.*, 1989; Connors *et al.*, 1992) resulting in low avidity antibodies to protective epitopes (Delgado *et al.,* 2009), poor Toll-like receptor (TLR) stimulation; suboptimal maturation of dendritic cells; decreased germinal center formation in lymph nodes; and the production of nonprotective antibodies (Ubol and Halstead, 2010).

Delgado et al., (2009) have attempted to fix the first generation FI-RSV by the incorporation of TLR agonists in adjuvants which later promotes dendritic cell maturation, T helper activation and B cell affinity maturation in Myd88+/- murine model but to what extent the deficiency in TLR activation which leads to the Th2 bias in the disease enhancement still remains to be answered. The authors suggested that if the FI-RSV can elicit high neutralizing antibodies against HRSV, the progression to

enhanced disease would be prevented in HRSV-naïve vaccinees upon subsequent wild type HRSV (wtHRSV) reinfection.

The role of the G glycoprotein as the cause of enhanced disease through the Th2 biased pathway have been suggested (Peter J. M. Openshaw *et al.*, 1992; G. E. Hancock *et al.*, 1996; Bembridge *et al.*, 1998; Teresa R. Johnson *et al.*, 1998) with Gs seems to play a significant role in eosinophilia compared to Gm (Bembridge *et al.*, 1998; Teresa R. Johnson *et al.*, 1998) upon challenge with wild type RSV. The epitope in the G glycoprotein which contributes to the Th2 biased response and eosinophilia had been mapped to aa184-198 (Tebbey *et al.*, 1998) and aa193-203 (Sparer *et al.*, 1998) corresponsing to the amino acid of the G glycoprotein of subgroup A HRSV. The inclusion of neutralizing anti-HSRV-G monoclonal antibody 1 day prior to challenge in FI-RSV vaccinated BALB/c mice demonstated the reduction of enhanced disease by 30% (Radu *et al.*, 2010), a practice which is not feasible in infants. However, enhanced disease was recently demonstrated by immunizing BALB/c mice with formalin inactivated recombinant RSV lacking the G glycoprotein (Polack *et al.*, 1999; Teresa R. Johnson *et al.*, 2004). Vaccination of BALB/c mice with Newcastle disease virus-like particles containing HRSV G glycoprotein demonstrated protection upon challenge with wtHRSV with no evidence of immunopathology (Murawski *et al.*, 2010).

1.7.2 **Live Attenuated HRSV vaccines**

As a next step into developing a safe and immunogenic HRSV vaccine, the feasibility of using live attenuated HRSV as a vaccine candidate was explored. The generation of live attenuated HRSV vaccine candidate involved serial passages of HRSV strain A2 in bovine cell line at low temperature and a cold passaged (cp) temperature sensitive (ts) RSV mutant, designated as RSV cpts530 was derived at passage 52 which is 10-fold less efficient in replication in mice compared to strain A2. Other group have reported the similar generation of cold passaged attenuated HRSV mutants by low temperature serial passages in Vero cells (Randolph *et al.*, 1994). Further attenuation was added to the cp mutants by mutagenesis with 5-fluorouracil rendering it more attenuated in primates and more stable *in vivo*. All the resulting mutants including cpts248/404, cpts248/955 and cpts530/1009 (with mutation at amino acid position 1009 in the polymerase) showed high attenuation in chimpanzees, immunogenic and confers significant protection upon challenged with wtHRSV (Crowe Jr *et al.*, 1994; Crowe *et al.*, 1995). Crowe et al., (1995) also reported that the administration of anti-HRSV

antibodies to chimpanzees prior to vaccination resulted in a significantly higher production of neutralizing antibodies to the challenged virus compared to the control group not receiving the immunoglobulins. Further attempts to attenuate the cold passaged mutants by recombinant means have yielded two candidates rA2cp248/404ΔSH and rA2cp248/404/1030ΔSH. Both candidates were highly attenuated in adults and in HRSV seropositive infants. They were immunogenic and well tolerated in HRSV naïve infants with the later exhibiting reduced replication of HRSV in naïve infants showing the important attenuating effect of missense mutation in aa1030 in the polymerase (Karron *et al.*, 2005). No disease enhancement was observed in all of the vaccinees although seroconversion upon wild type reinfection was low (20 of 54) in younger infants (1-3months) compared to (18 of 51) older infants. Nevertheless, virus shedding was negligible in vaccinees showing high attenuation of the vaccines and the low occurrence of URT disease among the vaccinees compared to the control group indicated the protective effect of the live attenuated vaccine (Wright *et al.*, 2007). Nevertheless, more work is needed to improve the immunogenicity as to obtain a higher seroconversion rate among vaccinees.

1.7.3 **Subunit vaccines**

The work on the development of anti-HRSV subunit vaccines concentrated mainly on the fusion glycoprotein due to its conservation between subgroups A and B. Nevertheless, a chimeric FG fusion had been described by expressing the fusion glycoprotein (aa1-489) as the N-terminus of the ectodomain of the G glycoprotein (aa97-279) using the baculovirus expression system (Wathen *et al.*, 1989b). The intramuscular immunization of mice with affinity purified FG subunit vaccine induced relatively low level of neutralizing antibody but surprisingly showed a marked >90% reduction in pulmonary HRSV upon intranasal challenge with wtHRSV (Prince *et al.*, 2000), a level which contradicts with the results previously reported (Connors *et al.*, 1992). Despite the impressive level of protection, immunized mice exhibited mild alveolitis and interstitial pneumonitis which can be eliminated by the addition of monophosphoryl lipid A (MPA) to the adjuvant. MPA is known to direct immune response to the Th1 pathway avoiding the Th2 biased pathway which is associated with the enhanced disease (Prince *et al.*, 2000). Nevertheless, these vaccine associated disease enhancement in parenteral administration of subunit FG or immunoaffinity purified F glycoprotein in cotton rats were not observed in cotton rats immunized with

vaccinia virus expressed recombinant HRSV fusion protein after challenged with wild type HRSV (Connors *et al.*, 1992).

Purified whole fusion glycoprotein (PFP) vaccine had been evaluated in clinical trials. PFP-1 and PFP-2 were prepared from HRSV strain A2 purified by immunoaffinity and ion exchange chromatography respectively (Dudas and Karron, 1998) and was shown to reduce the overall HRSV related incidence (Simoes *et al.*, 2001). PFP-2 had been shown to be safe and immunogenic in healthy institutionalized elderly (Ann R. Falsey, 1998), healthy adults over 60 years of age (Ann R. Falsey and Walsh, 1996) showing four-fold or higher rise in serum neutralizing antibody titer in 61% of the vaccinees. However frail institutionalized elderly showed reduced immunologic response to PFP-2 (Ann R. Falsey and Walsh, 1997). Trials on HRSV seropositive children shown that PFP-1 and PFP-2 are immunogenic without any disease enhancement upon wtHRSV challenge (Paradiso *et al.*, 1994; Piedra *et al.*, 1995; Jessie R. Groothuis *et al.*, 1998) but the neutralizing antibody titer was not long lasting and requires annual vaccination for continuous protection (Tristram *et al.*, 1994). No trials had been done on seronegative infants but maternal immunization with PFP-2 had been carried out in order to arm the newborn infant with high titered HRSV neutralizing antibody although the protective role of maternal antibody in protection against severe HRSV disease is still inconclusive. Nevertheless, pregnant women immunised with PFP-2 had HRSV specific antibodies detected in their expressed breast milk which might offer some degree of protection in breast fed infants (Janet Englund *et al.*, 1998). To minimize the possible risk of the subunit vaccine to the developing foetus, maternal vaccination was scheduled at the third trimester when the foetus is fully formed (Munoz and Englund, 2000) but this might prove to be suboptimal as the accumulation of IgG post infection happens in the first two trimesters and is suppressed in the third trimesters (Nandapalan *et al.*, 1986). Nevertheless, the inclusion of G glycoprotein with PFP in the subunit vaccine formulation showed marked increase in neutralizing antibodies suggesting the importance of G glycoprotein in the subunit vaccine strategy against HRSV (Gerald E. Hancock *et al.*, 2000).

The evaluation of the G glyocoprotein as a sole vaccine candidate had been evaluated. The amino acid 130-230 of HRSV strain A was fused to streptococcal protein G (BB) as a carrier protein and designated as BBG2Na.(Goetsch *et al.*, 2001). The administration of BBG2Na was not associated with enhanced disease upon challenge with wild type HRSV in mice (Plotnicky *et al.*, 2003; Power *et al.*, 2003) but low level of eosinophilia

had been reported in 2 of 4 seronegative macaques vaccinees (de Waal *et al.*, 2004). Parenteral immunization of healthy elderly elicited protective neutralizing antibodies which protected SCID mice from intranasal wild type challenge after passive transfer of antibody (Hélène Plotnicky-Gilquin *et al.*, 2002).

1.8 **Treatment**

1.8.1 **Ribavirin**

Ribavirin (Virazole® or 1-(β-D-Ribofuranosyl)-1*H*-1,2,4-triazole-3-carboxamide was licensed in the 1986 for the treatment of disease associated with the HRSV infection in hospitalized children (Ohmit *et al.*, 1996). Ribavirin acts by inhibiting the enzyme IMP dehydrogenase and the phosphorylated form inhibits the RNA polymerase (Nichols *et al.*, 2008).

The use of ribavirin remains controversial (Committee on Infectious Diseases, 1996) . Recent studies have shown that the administration of ribavirin during the HRSV bronchiolitis reduced the risk of asthma and allergen sensitization in high risk children but the length of hospitalization among the patients was significantly extended (Chien-Han Chen *et al.*, 2008) which agrees with the results of previous researchers (Ohmit *et al.*, 1996). However, the administration of aerosol ribavirin showed marked reduction in the duration of hospital stay (by 7 days) (Smith *et al.*, 1991) and reduction in the dependency for mechanical ventilation by 5 days for previously normal infants requiring mechanical ventilation due to severe HRSV infection (Hall *et al.*, 1983; Smith *et al.*, 1991).

Ribavirin had been administered to high risk adults with HRSV infections but the mortality rate remains high. The administration of intravenous ribavirin (IV-R) to HRSV infected bone marrow transplant (BMT) recipients is virtually ineffective and have been associated with 80% (2 of 10) mortality rate (Lewinsohn *et al.*, 1996) but the mortality rate was reduced to zero (0 of 10) when IV-R was co-administered with oral pnednisolone, a type of corticosteroid used in lung transplant recipients (Glanville *et al.*, 2005). The administration of aerosolized ribavirin to BMT recipients with HRSV related pneumonia resulted in approximately 28% (5 of 18) mortality (Hattington *et al.*, 1992) and 50% (3 of 6) mortality (Janet A. Englund *et al.*, 1988). The co-administration of aerosolised ribavirin with IVIG (RespiGam), an IgG preparation with high concentrations of HRSV neutralizing antibody to BMT recipients with HRSV related

pneumonia resulted in 9.1% (1 of 11) mortality (DeVincenzo *et al.*, 2000) and 6.25% (1 of 16) mortality (Small *et al.*, 2002). It is clear that ribavirin alone is poor in its efficacy as a prohylaxis against HRSV infection in the high risk groups unless supplemented with either anti-inflamatory corticosteroids or IVIG but IVIG alone is highly effective against HRSV infection. Following this, ribavirin is rarely administered to infants due to technical difficulty in delivery by aerosol inhalation (Clercq, 2004) coupled with the potential teratogenic effects of the drug (Lewinsohn *et al.*, 1996). Ribavirin has been associated with hepatoxicity in a stem cell transplant recipient with Hodgkin lymphoma suffering from HRSV infection suggesting a possible toxicity of ribavirin in immunocompromised adults (Chaves *et al.*, 2006).

1.8.2 **Immunoglobulin as prophylaxis**

The administration of intravenous immune globulin (IVIG) obtained from adult donors to high risk infants proved to be safe and effective in an outpatient setting but the titer of the HRSV neutralizing immunoglobulins are relatively low to offer prophylaxis against HRSV. The high HRSV neutralizing titer of IVIG found previously in individual IVIG preparation seems to be absent in 40 individual lots of IVIG from different suppliers suggesting the use of donor pools failed to ensure reproducibility, standard and efficacy of IVIG (J. R. Groothuis *et al.*, 1991). Due to this, high titered immune globulins (RSV-IG) were then prepared from adult human plasma with the minimum neutralizing titer three times higher (1:3000) than those in IVIG (1:1000). RSV-IG undergoes further processing and purification including steps to inactivate blood borne pathogens to make it more suitable for intravenous administration (Oertel, 1996). RSV-IG was approved by the US FDA in the early 1996 under the name RespiGam™ after the proven effectiveness in two major clinical trials involving National Institute of Allergy and Infectious Diseases (NIAID) (Ellenberg *et al.*, 1994) and Prophylaxis of Respiratory Syncytial Virus in Elevated risk Neonates (PREVENT). Although RespiGam was shown to be effective as passive prophylaxis in premature infants and infants under 2 years of age with bronchiopulmonary dysplasia (BPD) (Oertel, 1996), the risk of the transmission of blood borne viruses cannot be ruled out and obtaining reproducible batches of highly neutralizing antibodies from donor pools will prove to be a challenge.

Following this, the administration of anti-F monoclonal antibodies were shown to be protective, neutralizing, anti-fusogenic and eliminates virus from lungs of infected mice and cotton rats (G. Taylor *et al.*, 1984; Edward E. Walsh *et al.*, 1987; Routledge *et al.*,

1988; G. Taylor *et al.*, 1992; Skaricic *et al.*, 2008) and this passive protective effect was also observed in the gnotobiotic calves against BRSV infection (Thomas *et al.*, 1998). To this a humanised murine monoclonal antibody palivizumab (Synagis, Abbott, UK) was developed. Palivizumab is recombinant based, eliminating the risk of blood borne virus transmission and ensured highly reproducibility between batches. Palivizumab is the only monoclonal antibody licenced for prophylaxis of HRSV (Sandritter, 1999; Herren Wu *et al.*, 2007a) and can be delivered through intramuscular route rather than intravenous as with RespiGam™. The efficacy of Palivizumab was demonstrated in a large clinical trial, known as The Impact RSV Study Group, (1998) which involved 1502 children with prematurity or with BPD. The study showed reduced hospitalization rates of HRSV high risk groups following the administration of intramuscular Palivizumab. These studies also reported that infants receiving Palivizumab had reduced HRSV hospital days, oxygen supplementation and moderate to severe lower respiratory tract infection (LRTI) (Thwaites *et al.*, 2008). However, the administration of Palivizumab only reduced the hospitalization rate by 55% (Group, 1998) and 45% (Feltes *et al.*, 2003) with no significant reduction in the rate of mechanical ventilation or length of stay in the Intensive Care Unit (ICU). This raised the question of the cost effectiveness and efficacy of Palivizumab (Landau, 2006).

Recently, Palivizumab resistant HRSV strains were isolated from infants receiving Palivizumab treatment and their characterization revealed a mutation in codon 272 and 276 in the F gene in which resulted in a complete loss of binding to Palivizumab (Adams *et al.*, 2010). Also, HRSV from nasopharyngeal secretions and low passages of virus isolates have been was shown to be significantly resistant to neutralization by F protein specific monoclonal antibodies including palivizumab (Marsh *et al.*, 2007). These resistant viruses replicated poorly in cell culture and upon cell culture adaptation, the higher passage viruses were shown to increase in susceptibility to Palivizumab without any change of the binding of Palivizumab to the isolated F protein in BIAcore™ assay (Marsh *et al.*, 2007).

A second generation immune prophylaxis involving the affinity maturation of humanized monoclonal antibodies derived from Palivizumab has been developed. This humanized monoclonal named motavizumab (MEDI-524, Numax) developed by MedImmune Inc., a subsidiary of AstraZeneca targets antigenic site A of the F glycoprotein only differs from its predecessor by 13 amino acids. Motavizumab shows approximately 20-fold improvement in virus neutralization *in vitro* and up to 100-fold

reduction in virus titer in cotton rats compared to Palivizumab at equivalent concentration. Unlike Palivizumab, motavizumab is potent in the inhibition of viral replication in the upper respiratory tract (Herren Wu *et al.*, 2007a). Motavizumab exhibited 70-fold enhancement in binding to the F glycoprotein, 11-fold faster association and 6-fold slower dissociation rate compared to Palivizumab (Weisman, 2009). Clinical trials have shown that motavizumab to be comparable to Palivizumab in both safety and efficacy in the reduction of incidence of RSV-related hospitalizations. A detailed summary of the progression of the clinical trials from phase1/2 to phase 3 was reviewed by Cingoz (2009). However, motavizumab was not approved by the US FDA and the licensing application had been withdrawn by AstraZeneca.

Additional work on passive immunization of Mabs to the G glycoprotein showed similar protective effect in mice and cotton rats (G. Taylor *et al.*, 1984; E E Walsh *et al.*, 1984; Stott *et al.*, 1986; Routledge *et al.*, 1988) but most anti-G Mabs especially those recognizing subgroup specific epitopes involving the conserved cysteine noose region of the G glycoprotein do not neutralize the virus *in vitro* (Trudel *et al.*, 1991; Simard *et al.*, 1997; Helene Plotnicky-Gilquin *et al.*, 1999) and significantly less effective than anti-F Mabs. Mekseepralard et al. (2006b) by using a aglycosyl mutant to the C_H2 domain which renders the antibody defective in complement activation and FcγR binding have shown the reduced protective effect of a subgroup specific monoclonal antibody in BALB/c mice upon challenged with HRSV showing the complex involvement of both Fc and non-Fc mediated interactions in protection.

Chapter 2 Background of project

2.1 **Introduction**

Pneumonia is the leading cause of mortality in children below five years old which accounts for 1.6 million children annually or 18% of the total number of children in that age group worldwide with the main viral etiological agent being human respiratory syncytial virus (WHO, 2010). As reviewed above, the only treatments licensed to treat or prevent HRSV related respiratory illneses are the administration of aerosolized ribavirin and/or intramuscular administration of Palivizumab but the efficacy and the reduction of hospital stay associated with the treatments are not satisfactory (Committee on Infectious Diseases, 1996; Landau, 2006). It is possible that the administration of a single monoclonal antibody targeting the highly conserved fusion protein alone as a means of prophylaxis is insufficient *in vivo* and the inclusion of anti-G monoclonal antibody specific to the contemporary genotype or cross reactive as a complement to Palivizumab may boost the efficacy of Palivizumab in reducing the hospital stay and duration of dependency on mechanical ventilation.

The F glycoprotein exhibits a lower evolutionary rate compared to the G glycoprotein as reviewed in section 1.5.1. The relative conservation of the fusion glycoprotein suggests that the fusion glycoprotein either cannot tolerate changes or is a relatively unimportant antigen compared to the G glycoprotein (Xiaodong Zhao *et al.*, 2004). In line with the latter possibility, escape mutations occur in the F gene and can be readily selected by exposure of the virus to anti-F antibody in cell culture systems. Prolonged treatment of a 4 year old girl with severe combined immune deficiency disease (SCID) with IVIG resulted in the emergence of an escape mutant carrying a novel mutation to the G glycoprotein but not the F glycoprotein (Lazar *et al.*, 2006).

The finding that antibodies to the F glycoprotein can be protective in infants favours the first alternative, F is an important antigen but is unable to vary antigenically. However, the facile creation of antigenic variations in F in culture under immune pressure argues for the alternative hypothesis that F cannot change and must therefore not be under immune pressure *in vivo*. These apparently conflicting views can be resolved if anti-F antibodies are important in protecting the infant lung but unimportant in preventing transmission of the virus from the upper respiratory tract. Immune pressure is exerted on virus transmission, selecting escape mutants able to carry infection from one individual to another. The ability of anti-F antibodies to protect the infant lung is irrelevant to transmission.

If anti-G antibodies are important in controlling transmission of HRSV in the population resulting in a high evolutionary rate and emergence of G glycoprotein antigenic variants, anti-G antibodies may also play a role in protecting the infant lung, and therefore might have prophylactic potential.

In an epidemiological study Scott et al., (2006) showed that infants with primary HRSV infection lost their subgroup specific immunity 2-4 months after infection but retained their infecting genotype specific immunity for up to 7-9 month suggesting that genotype specific antibodies, which are mainly anti-G antibodies following primary infection in infancy (McGill *et al.,*2004b) are protective longer than group specific antibodies, which are predominantly anti-F and to a lesser extent anti-G.

Antibodies elicited against the G glycoprotein of HRSV can be either subgroup specific, cross-reactive or genotype/strain specific, targetting different regions of the G glycoprotein as depicted in Figure 5 in section 1.4.4. Nearly 45% of adults shown seroconversion to the central unglycosylated region of G which contains cross-reactive epitopes (Murata et al., 2010) and these antibodies are therefore likely to be passed across the placenta to at least a proportion of infants. It has not been possible, hitherto, to assess the importance of cross-reactive anti-G antibodies in protection against primary HRSV. However, demonstration of the presence of high titered cross-reative and protective maternal antibody would indicate the possibility of designing a single potent pan-HRSV vaccine or immunoprophylaxis against the G glycoprotein. Previous conclusions that anti-F antibodies are protective in infants were derived from the correlation of maternal antibody level to the membrane bound glycoproteins at the time of infection with the evidence of HRSV infection (Glezen *et al.*, 1981b; Ogilvie *et al.*, 1981). Kasel et al., (1987) also showed a significant correlation of anti-F but not anti-G antibodies with immunity although there was no significant correlation between reduced severity of illness among hospitalized infants and antibody to the purified glycoproteins of the subgroup A genotype of HRSV (Toms *et al.*, 1989). In the latter studies prototype strains (A2 and Long) were employed, which were isolated about two decades previously. It is likely that only an undefined fraction of the anti-G antibodies would have been detected in contemporary sera by these antigens potentially masking any protective effect of anti-G antibodies. Similar studies, aiming to demonstrate a correlation of anti-G antibodies with reduced incidence of HRSV bronchiolitis, must necessarily measure anti-G antibodies against contemporary genotypes of the virus utilising assays which measure either all antibody species reacting with the G

glycoprotein of the infecting strain or, to assess the role of cross-reactive antibodies, exclusively all of the antibody species reacting with the conserved epitopes of the G glycoprotein.

2.2 **Hypothesis**

Anti-G antibodies, measured against the G glycoprotein of the infecting virus genotype, are important in the protection of infants against severe HRSVdiseases.

2.3 **Aims**

I. To establish the molecular epidemiology of HRSV in Newcastle upon Tyne over three seasons. Molecular epidemiology by phylogenetic analysis will accurately define the contemporary genotypes and predict the genotypes in the subsequent epidemic. This will also provide contemporary genotypes of HRSV for the expression of F and G glycoproteins. The utilization of contemporary genotypes to generate recombinant glycoproteins is preferable as previous research has shown that antibodies in covalescent sera exhibit reduced binding to the fusion glycoprotein derived from strain A2 compared to contemporary viruses of heterologous genotypes.

II. To isolate and express the F and G glycoprotein genes of contemporary HRSV strains and to develop a lectin capture ELISA to measure anti-F and total anti-G antibodies to the infecting virus genotype in infant sera.

III. To develop an assay for the measurement of antibody exclusively to the conserved epitopes of the G glycoprotein.

IV. To compare anti-G glycoprotein maternal antibody levels between hospitalized infants with severe HRSV disease and a comparison group of uninfected infants hospitalized with no evidence of HRSV infection to test the hypothesis that anti-G glycoprotein antibodies correlate with protection against severe HRSV disease.

Chapter 3 Materials and methods

3.1 General

3.1.1 Materials

All chemicals were from SIGMA unless stated otherwise.

All chemicals, reagents, plasticware and glassware are sterile unless stated otherwise.

3.1.2 Sterilization

All sterilizations were carried out in an autoclave machine (AMA252, Astell Scientific Ltd) with moist heat at 121 °C for 15 minutes unless stated otherwise.

3.1.3 Water

All tissue culture reagents were prepared and diluted in Milli-Q water, Milli-Q synthesis (Millipore).

Non-tissue culture related reagents and chemicals were prepared and diluted in distilled water.

3.1.4 Surface Decontaminations

Work surfaces were decontaminated before and after experiments with either Virkon or Trigene Advance. Surface was wet with the decontamination solution and wipe dry with paper towel.

3.1.5 Incubation

All incubations in this section was done in a moisture enhanced incubator supplemented with 5 % CO_2 at 37 °C.

3.2 Cell count

Trypsinised cells were resuspended in λ ml of medium and 15-20 µl was spotted onto an Improved Neubauer Haemocytometer. The total number of cells in all four cell corners were counted (β cells).

The cell concentration, $M_{cell} = \frac{\beta}{4} \times 10^4$ cells/ml

Total cells per flask, $\sum \boldsymbol{cell} = \frac{\boldsymbol{\beta}}{\boldsymbol{4}} \times 10^4 \times \lambda$.

3.3 Preparation of Chicken cell fibroblast (CEF)

Ten fertilized hen's eggs were grown at 38 °C for 10 days in a moisture supplemented incubator with twice daily rotation. The eggs were sterilized with methanol in the tissue culture hood and air dried. Sterile sharp scissors were used to stab open the egg over the air sac and the shell was carefully cut away. The membranes were peeled back using a curved forceps and the embryo was lifted out from the egg. The embryos were decapitated and placed in a 50 ml centrifuge tube containing 20 ml of embryo washing buffer to remove erythrocytes from the embryo. All the embryos were rinsed further with embryo washing buffer and placed into the barrel of a 20 ml syringe, and forced through with the plunger into a Duran bottle containing 200 ml of digestion buffer and incubated at room temperature for 30 minutes with gentle stirring with a sterile magnetic stirrer. The stirring was stopped and the suspension kept still for 5 minutes to sediment large undigested fragments. The suspension was then transferred into 50 ml centrifuge tube containing 5 ml of FCS to inactivate the trypsin before being filtered through a sintered glass filter (No 3). The flow through was spun at 1000 rpm for 10 minutes and the pellet was resuspended in 10 ml of GM1. The cell concentration was adjusted to 10^7 cells/ml in cryo buffer and frozen down at 1°C/hour in "MrFrosty" (Cat~5100-0001) (Nalgene) at -80 °C freezer for 16 hours and stored in liquid nitrogen until use.

3.4 Human epithelial carcinoma cell line (HeLa)

HeLa cell line was obtained from Dr. Sarah Elizabeth Welsh, Newcastle University, UK without any recorded passage number. This cell line has been in continuous use for the isolation and culture of HRSV in Newcastle upon Tyne virus diagnostic laboratories, Royal Victoria Infirmary for at least 34 years (Herrnstadt *et al.*, 2002).

3.5 Japanese Quail fibrosarcoma cell line (QT35)

QT35, passage 26 was a gift from Dr Barbara Blacklaw, Cambridge University.

3.6 Baby Hamster Kidney cell line (BHK21)

BHK21 was a gift from Dr C.R Madeley, Health Protection Agency, Newcastle upon Tyne.

3.7 **Routine cell culture**

All routine cell culture methods described in this section are identical to all cell lines unless stated otherwise.

3.7.1 **Maintenance of stock**

The medium from a confluent monolayer in a T75 tissue culture flask was aspirated and rinsed once with 5 ml PBS. The monolayer was treated with 5 ml of Trypsin/versene and 4 ml was removed followed by incubation at 37 °C for 5 minutes, the flask was knocked a few times to dislodge the cells which were then resuspended in 5 ml of the appropriate growth medium (GM). A third of the volume was transferred into a new flask and volume of GM adjusted to 10 ml. GM was replaced every two days.

HeLa cells were grown in 4 oz glass bottles and maintained as described above.

3.7.2 **6 well plate**

Each well was seeded with 1.6×10^6 cells/well in 2 ml of GM and incubated overnight to achieve a confluent monolayer the next day.

OR

One newly confluent cell monolayer in T75 tissue culture flask was trypsinised as described above and resuspended in 26 ml of GM and seeded 2 ml/well for 2 plates followed by overnight incubation to achieve a confluent monolayer the next day.

3.7.3 **24 well plate**

Each well was seeded with 4×10^5 cells/well in GM and incubated overnight to achieve a confluent monolayer the next day.

OR

One newly confluent cell monolayer in T75 was resuspended in 50 ml of GM and seed 1ml/well for 2 plates and incubated overnight to achieve a confluent monolayer the next day.

3.7.4 **96 well plate**

HeLa cells grown in 96 well plates were used for HRSV virus titration. One newly confluent HeLa cell monolayer in 9 oz glass bottle were resuspended in 50 ml of GM and seed 200 µl/well and incubated overnight to achieve a confluent monolayer the following day.

3.7.5 **HeLa tubes**

In the morning when HRSV isolation is to be carried out, 1 flask of confluent HeLa cells in 4 oz glass bottle was dissociated as described above and resuspended in 18ml of GM2 and used to seed 1ml/tube in glass test tubes. The test tubes were placed on a test tube rack, sealed with a sterile silicone bung and incubated with approximately 10° elevations ensuring that the medium does not wet the bung. After approximately 6 hours of incubation, the monolayer should achieve 50-60 % confluency and are ready to be used.

3.8 **Diff-quik Staining**

The nucleus and cytoplasm of cell monolayer was stained with Diff-quik® (Cat#F07528/71) (Dade Behring). The monolayer in 24 well plate was rinsed with 500 µl/well of PBS and fixed with 200 µl/well of Fixative Solution [0.002 g/L (w/v) Fast green in Methanol] by dispensing the volume onto the cell and immediately removed by aspiration. This procedure was repeated for 3 times. The fixed monolayer was air dried and stained with 200 µl/well of Stain Solution I [1.22 g/L (w/v) Eosin G in phosphate buffer, pH 6.6] as described above and followed with Stain Solution II [1.1 g/L (w/v) Thiazine Dye in phosphate buffer, pH 6.6]. The stained monolayer was washed once with 500 µl/well of distilled water, air dried and visualized under the microscope.

3.9 **Immunological reagents**

Table 2 Lists of primary antibodies

Antibodies	Antigen/Specificity	Concentrationφ (µg/ml)	References
HRSV Mab pool	HRSV F, M2, P and N	N/A	Novocastra Laboratories
1A12	HRSV F	573	(Routledge *et al.*, 1985)
IC2	HRSV G Subgroup A	540	(Morgan *et al.*, 1987)
133	HRSV G Conserved	25	(M. J. Robinson, 2007)
21	HRSV G Conserved	15.8	(M. J. Robinson, 2007)
4G4	HRSV G Subgroup A	19.6	(Morgan *et al.*, 1987)
3F43	HRSV G Strain A2 specific	600	(Morgan *et al.*, 1987)

φ concentrations were determined by Ms Fiona Fenwick, Newcastle University

Table 3 Secondary antibodies

Name	Abbreviation	Dilution	Reference
Goat anti-mouse-HRPψ Cat#NCL-GAMP	GAMPx	1/1000φ	Novocastra
Polyclonal Rabbit anti-human IgA-HRP Cat#P216	RAHAPx	1/200φ	Dako
Polyclonal Rabbit anti-human IgG-HRP Cat#P0406	RAHGPx	1/800φ	Dako
Sheep anti-mouse FITC (Novocastra)	SAM-FITC	1/20γ	Novocastra

φ Dilution in PTF, γ diluted in Evan's blue counterstain, ψ Horse radish peroxidise conjugate

3.9.1 **Cord blood**

Cord blood from 17 donors was obtained from archived frozen stock stored at -20 °C collected in 1980/1981 by Dr. Ron Scott.

3.10 **Immunofluorescence**

3.10.1 **Microscope slides**

All PTFE coated multispot microscope slides (Hendley Ltd) were treated with 10 % (v/v) Decon90 (Decon Laboratories Ltd) in distilled water for 2 hours and washed with running tap water for 4 hours. Slides were soaked in distilled water overnight, air dried and stored at ambient temperature until use.

3.10.2 **Slide preparation**

Monolayers from the tissue culture flasks or plates were scraped into the original volume of the appropriate maintenance medium with a a cell scraper (Corning) and 15 to 20 µl of the cell suspension was spotted onto microscope slide (section 3.10.2), air dried in a biological safety cabinet.

3.10.3 **Specimen fixation**

The dried specimens were fixed by immersing specimen slides into ice-cold acetone for 10 minutes and then air dried. The slides were either used immediately or stored at -20 °C until use.

3.10.4 **Indirect immunofluorescent staining**

The slides were equilibrated to ambient temperature and air dried if water of condensation occured. 10 µl of the primary antibody was spotted onto the fixed cells and incubated for 30 minutes in a moist chamber at 37 °C. The primary antibody was removed by immersing the slide in PBS for 5 minutes and the slide was air dried. 10 µl of SAM-FITC antibody and spotted onto the primary antibody stained cells and incubated for 30 minutes and then washed as above. The spots on the slide were covered with a drop of immersion oil (Cat# 16212) (Cargille Laboratories Ltd) visualized via a 50X oil immersion lens (Nikon Plan) in a fluorescence microscope Eclipse E400 (Nikon). The intensity of the fluorescence was recorded based on arbitrary four scale fluorescence with 1+ being the lowest and 4+ being the highest. Photographs were captured using a built-in Coolpix 4500 4 megapixels digital camera (Nikon).

3.11 **ELISA**

3.11.1 **ELISA plates**

F96 Maxisorp Nunc-immunoplate (Cat#442404) (Thermo) was used and refered to as Maxisorp plates.

3.11.2 **Washing**

Wells of the Maxisorp plate was filled with the appropriate wash buffer, decanted and blot dried. This process is repeated trice for each washing.

3.11.3 **Incubation**

All incubation was done at 37 °C with the Maxisorp plate covered with a plastic lid to minimize evaporation.

3.11.4 **Preparation of HRSV ELISA antigens**

The maintenance medium was removed from 4 infected 4oz bottles of HeLa monolayers exhibiting 3+ to 4+ CPE and replaced with SAF. Cultures were incubated for a further 16 hours before cells were scraped into the medium and the cell suspension was pooled into a 50 ml sterile centrifuge tube and pelleted by centrifugation at 1000 rpm for 5 minutes. The pellet was resuspended in 2 ml of ice cold SAF and sonicated on ice using a SANYO MSE Soniprep sonicator, with amplitude of 10 microns, 3x30 seconds bursts with 30 seconds interval between each burst. The sonicated cell lysate was snap frozen in liquid nitrogen, thawed and aliquoted as 200 µl aliquots, snap frozen and stored at -80 °C until use. A parallel experiment was carried out with uninfected HeLa cell control.

The total protein concentrations for the virus lysate and cell control antigens were estimated as described in section 3.14.

3.11.5 **Preparation of recombinant ELISA antigens**

Four flasks of confluent HeLa in T225 were prepared by seeding one confluent T75 flask of HeLa into one T225 flask and cultivated in 40 ml of growth medium per flask. The growth medium was removed by aspiration and inoculated with MVA adjusted to the MOI of 3 in 5 ml of MM2 an incubated for 1 hour. 20 ml of fresh MM2 was added

and incubated for 48 hours. The cells should show 4+ CPE and most have detached from the flask into the medium.

Two flasks worth of monolayer was scraped into the medium and transferred into a 50 ml centrifuge tube and pelleted by centrifugation at 1000 rpm for 5 minutes. The medium was removed by aspiration and the pellet resuspended in 50 ml of PBS and held on ice for 5 minutes. The cell was pelleted by centrifugation was above and PBS removed by aspiration. The cells were resuspended in 6 ml of ice cold lysis buffer prepared as described in section 3.13 and sonicated on ice bath 3 x 30 seconds at 10 microns with 30 seconds interval using Sanyo MSE sonicator. The lysed antigen was stored as 500 µl aliquot in 2 ml vial at -20 °C until use.

3.11.6 **Direct ELISA protocol**

HRSV virus antigen and uninfected cell antigens were adjusted to 10 mg/ml in SAF and dilutions were made in 1X bicarbonate coating buffer to the desired dilutions. Half of the plate was coated with HRSV virus antigen and the other half with uninfected cell antigens at the volume of 50 µl/well followed by overnight incubation.

The following day, unbound antigens were decanted and washed. No dedicated blocking step was carried out unless stated otherwise. The Maxisorp plate was either used immediately or stored frozen at -20 °C.

Primary antibody was diluted in PTF to the desired dilutions and dispensed at the volume of 25 µl/well. The plate was incubated for 90 minutes followed by washing step with PBST.

The primary antibody stained wells were then stained with the appropriate secondary antibody dilution and incubated for 60 minutes followed by washing step with PBST.

Colour was developed using 50 µl/well of OPD and incubated for 30 minutes. Reaction was stopped with 50 µl/well of 3M H_2SO_4 and absorbance read at 492 nm in an ELISA plate reader.

3.11.7 **ConA capture ELISA**

Stock ConA prepared as described in section 12.2.4 was diluted to 50 µg/ml in PBS and coated on Maxisorp plate at 50 µl/well followed by overnight incubation.

Excess ConA was decanted, washed with PBSTx and blocked for an hour with 100 µl/well of PTF. PTF was decanted and washed with PBST.

The subsequent steps from the primary antibody staining onwards were carried out as described in section 3.11.6.

3.11.8 **Additive binding ELISA**

HRSV antigen was titrated twofold horizontally in BCB on a Maxisorp plate and incubated at 37 °C for 2 hours. Uninfected HeLa cell antigen was coated in parallel. The Maxisorp plate was washed and monoclonal antibody was twofold titrated vertically in PTF and direct ELISA was performed as described in section 3.11.6 using GAMPx as the secondary antibody. The saturating antigen concentration with the excess antibody dilution was chosen for the additive binding ELISA.

Half of a Maxisorp plate was coated with the saturating HRSV antigen concentration and the other half with the equivalent uninifected HeLa cell concentration. The monoclonals were assayed individually as a control. Additive binding ELISA was prepared by diluting the antibody in PTF yielding double the desired concentration and combined at equal volume to get the test dilution. ELISA was carried out described in section 3.11.6 with GAMPx as the secondary antibody.

3.12 **SDS-PAGE and Western blot**

3.12.1 **Preparation of polyacrylamide gel**

BIORAD cell II system was used throughout. The glass slab was assembled with 0.1 mm spacer on the cast system and each glass slab was filled with 5 ml of 10 % resolving gel formulation and carefully overlayed with distilled water and let to polymerise at 37 °C for 1 hour. Water overlay was decanted, rinsed with distilled water and blot dry with filter paper. The polymerised resolving gel was overlayed with 5 % stacking gel and comb was inserted and polymerised at 37 °C for 30 minutes. The comb was removed by lifting it vertically out from the gel and the wells were rinsed vigourously with distilled water.

The gel cast was then transferred onto the electrophoresis set and filled with ice cold 1X electrode running buffer.

3.12.2 **Protein sample preparation**

Protein sample was diluted in 4X reducing sample buffer (150 µl of protein plus 50 µl of 4X reducing sample buffer), vortex mixed and boiled for 10 minutes in a boiling waterbath. The protein sample was immediately cooled on ice for 1 minute followed by centrifugation at 13000 rpm for 10 minutes in a microfuge to pellet any insoluble protein particles.

3.12.3 **SDS-PAGE**

Protein samples prepared in section 3.12.2 was loaded into a well in the polyacrylamide gel (section 3.12.1) and resolved at 120V until the bromophenol blue dye front reaches about 1cm from the end of the gel.

3.12.4 **Transfer of SDS PAGE gel.**

Hybond-P Polyvinylidene fluoride (PVDF) transfer membrane (GE Healthcare) was cut to the size of the SDS-PAGE gel and soaked in methanol for 1 minutes and stored in ice cold 1X Towbin buffer until use.

The glass plates' holding the SDS-PAGE gel was removed from the electrophoresis set and a glass plate was removed exposing the gel. The gel was rinsed with ice cold 1X Towbin buffer and sandwiched together in a Biorad transfer apparatus with 1 sheet of

Whatman filter paper and one sponge in either side of them and inserted into the electrophoresis cell. An ice pack was inserted and the electrophoresis set was flooded with ice cold 1X Towbin buffer and transfer was done at 100V for 1 hour. The temperature of the electrophoresis cell was regulated with the help of contant stirring with a magnetic stirrer. The membrane was removed from the set and blocked overnight in blocking buffer.

3.12.5 **Western blots staining**

The membrane resulting from section 3.12.4 was incubated for 1 hour with 10 ml of primary antibody diluted in blocking buffer in a 50 ml centrifuge tube on a rotating mixer. The membrane was washed 3 times in PBST and subsequently incubated with GAMPx in blocking buffer and incubated as above in a fresh 50 ml centrifuge tube. The membrane was washed three times in PBST, dried on a piece of filter paper and stained in 20 ml of Substrate A and B n the dark for 1 hour on an orbital shaker. The membrane was soaked in distilled water overnight and air dried.

3.13 **Modified Lowry**

This method was originally described by (Lowry *et al.*, 1951) and modified by (Markwell *et al.*, 1978).

3.13.1 **Standard curve**

Due to the presence of Triton X-100 in PBSTx used to extract the fusion glycoprotein, recombinant antigens cannot be estimated by using the Biorad protein Assay. Thus Modified Lowry Assay (Markwell *et al.*, 1978) was chosen for the estimation of total protein from the assay as described in section 3.13.2.

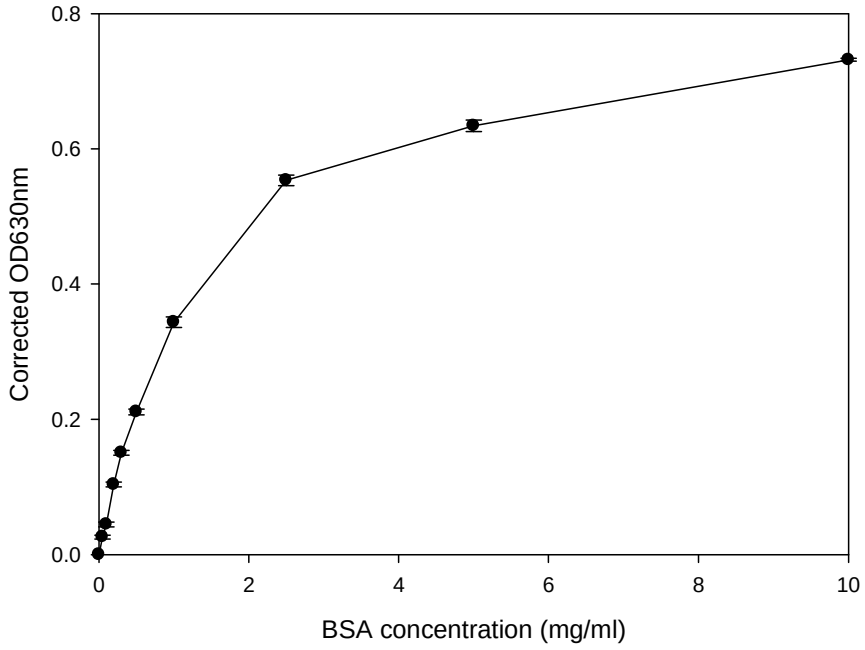


Figure 11 Standard curve for BSA based on modified Lowry protein assay.

A BSA standard curve was prepared from 10 mg/ml down to 0 mg/ml and assayed as described in modified Lowry Assay. From the standard curve in Figure 11, the curve shows two linear ranges between 0-0.5 mg/ml and 5-10 mg/ml. Due to practicality, BSA standard curve was prepared within the first linear range and graph ploted in Microsoft Excel 2007 with equation displayed for calculation.

Proteins assayed was serially diluted in PBSTx and assayed together with the standard. The actual concentration was calculated based on the lowest dilution where the optical density falls into the linear range of the standards.

3.13.2 **Procedure**

100 µl of standard/sample was aliquoted into a fresh ependorf tube and combined with 300 µl of Reagent C. The mixture was vortex mixed and incubated for 30 minutes at 37 °C in an incubator. 30 µl of Reagent D was added, vortex mixed and incubated for 30 minutes at 37 °C in an incubator. The mixture was dispensed at 100 µl/well into a 96 well plate and read at 630 nm on a ELISA plate reader.

3.14 **Biorad Protein Assay**

3.14.1 **Procedure**

10 µl of protein sample was diluted to 150 µl with 0.1 % (w/v) SDS and dispensed with equal volume of working Biorad Assay Reagent, mixed well by pipetting and optical density read at 600 nm in a microwell plate reader. The protein standard was treated in parallel and a protein standard curve was ploted in Microsoft Excel 2007 and equation was displayed for calculation.

3.15 **Molecular biology**

3.15.1 **Oligonucleotides**

All primers were synthesized by Eurofins MWG Operon. All cloning oligonucleotides were high performance liquid chromatography (HPLC) purified and all regular PCR primers were high purity salt free (HPSF) purified.

Table 4 Oligonucotides used for genotyping

Primer	Sequence	Polarity	Location	Reference
GC1	GCAGCATATGCAGCAACA	+	533–551α	(Roca *et al.*, 2001)
F164	GTTATGACACTGGTATACCAACC	-	186–164α	(W. M. Sullender *et al.*, 1993)
GB1	CCTGCAGGCAATGATAATCTCAACCTC	+	153-173β	(A. McGill, 2001)
GB2	GAATTCTCGGAGTGGAGGGATTTGCTGTTGG	-	818-798β	(A. McGill, 2001)
G1	(GGATCCC)GGGGCAAATGCAAACATGTCC	+	1-21α	(P. A. Cane and Pringle, 1992)

α sequence corresponding to strain A2, β sequence corresponding to strain 8/60, +and – polarity also represents forward primer and reverse primer respectively.

Table 5 Oligonucleotides used for cloning G genes and F gene into pSC11

Primer	Sequence (5'-3')	Group	Gene
gpGA_For	TAACCCCGGGGCAATTGCAAACATGTCCAAA	A	G
gpGA_Rev	TGGTCCCGGGTTT*CCTCAGG*AATACGCTTTTTAATGACTA	A	G
gpGB_For	TCAAAAACAACCCCGGGGCAATTGCAAACATGTCCAAA	B	G
gpGB_Rev	TCTCTGCCCCGGGGTA*CCTCAGG*AATAACTAAGCATATGACT	B	G
gpFA_For	TCAACCCCGGGGCAAATAACAATGGAGTTGCCAATCCTC	A	F
gpFA_Rev	ATGACCCCGGG*CCTAAGG*CATTGTAAGAACATGATTAGGTGC	A	F

Underlined: Cfr9I RE site, Boxed: ATG transcription start codon, italised: BSu36I RE cleavage site.

Table 6 Oligonucleotides used in colony PCR

Primer	Sequence (5'-3')	Vector
M13_For	CGCCAGGGTTTTCCCAGTCACGAC	pGEM-T Easy
M13_Rev	TCACACAGGAAACAGCTATGAC	pGEM-T Easy
pSC_For	TATACATAAACTGATCACTAATTCC	pSC11
pSC_Rev	TTGAAATGTCCCATCGAGTG	pSC11
pTM_coFor	TCGGTGCACATGCTTTACAT	pTM3
pTM_coRev	CCCCAAGGGGTTATGCTAGT	pTM3

Table 7 Oligonucleotides used for the design of the colony PCR primer

Primer	Sequence (5'-3')
SH Forward	CGCATAGCCGGCATGAAAACATTAGATGTCATAAAAAG TGATG
SH Reverse	CGCATAGCCGGCCCTGAGGCGAATGTTCTCCACTCTTAC TTC
MCS_upstream	CATCACTTTTTATGACATCTAATGTTTTCAT
MCS_downstream	GAAGTAAGAGTGGAGAACATTCGCCTCAGG
pTMvec_F	CCG GGGAGCTCACTAGTGGATCCCTGCAG
pTMvec_R	CTGCAGGGATCCACTAGTGAGCTCCCCGG

Table 8. Oligonucleotides used in the for the cloning of the G gene and its truncated products into pTM3

Primer	Sequence (5'-3')
GNcoIF	CGCAATTGCAACCATGGCCAAAACCAA
G-N151F	AAGTCCACCACCATGGAACGCCAAAACAAACCACCAAAT
G-N155F	AAACAACGCCCCATGGAACCACCAAATAAACCCAACAAT
G-N158F	CAAAACAAACCCATGGATAAACCCAACAATGATTTTCAC
G284R	TGTGAT*GGATCC*TCAGATGTTGTATAGAC
G190R	CTTTCCAGG*GGATCC*TCATGGTATTCTTTTGCAGAT
G-177R	CCAGCAGGT*GGATCC*CTAGCATATGCTGCAGGGTACAAA
G-172R	GTTGCTGCA*GGATCC*CTAGGGTACAAAGTTGAACACTTC
G163R	GTTGAACAC*GGATCC*TCAAAAATCATTGTTGGGTTTA

Boxed-transcriptional start codon which is also part of the engineered NcoI restriction site, Italicised-engineered BamHI restriction site, Underlined-engineered stop codon.

3.15.2 **RNA extraction**

RNA extraction was performed using the RNeasy Mini Kit (Cat#74106) (Qiagen) with modification where virus stock in maintenance medium was used instead of serum free infected cell pellet. Three buffers namely Buffer RLT, RW1, RPE and RNase free water were supplied with with the kit.

500 µl of frozen virus stock was thawed in 1 volume of Buffer RLT (lysis buffer) followed by the addition of 1 volume if 70 % (v/v) ethanol diluted in DEPC water. The lysate was filtered through an RNeasy mini column by brief centrifugation and washed once with 700 µl of buffer RW1 and twice with 500 µl of Buffer RPE. The total RNA was eluted in 2x30 µl of RNase free water supplemented with 60 units of Ribolock™RNase inhibitor (Cat#EO0381).

HeLa cell was concurrently extracted with the virus stock as a cell control. Maximum of 9 reactions were permitted at any one time for optimal loading into the centrifuge.

3.15.3 **Reverse transcription**

All reagents in this section were purchased from Fermentas unless stated otherwise. The first strand cDNA synthesis was carried out in 30µl reaction volume in a 24 well PCR plate containing 5 mM $MgCl_2$, 1mM of each dNTP,

20 pmol of forward primer,

40 units of Ribolock™ RNase Inhibitor (Cat#EO0381),

200 units of RevertAid™ H Minus Reverse Transcriptase (Cat#EP0451),

1X reverse transcription buffer

10 µl of total RNA extract

The reaction was carried out in a PTC-200 Peltier thermal cycler (M.J. Research Inc.) at 42 °C for 30 minutes followed by a denaturation step at 99 °C for 5 minutes. The cDNA was used immediately or being stored at -20 °C.

3.15.4 **Polymerase Chain Reaction**

All reagents were purchased from Bioline unless stated otherwise.

All PCR reactions use identical formulations as described below. The reaction was carried out in a 100 µl reaction volume in a 24 well PCR plate containing

10 µl of cDNA (section 3.15.2)

10 µl of the 10X PCR buffer

0.25 mM of each dNTP

20 pmol/µl of forward primer

20 pmol/µl of reverse primer

4 mM of $MgCl_2$

0.5 unit of Immolase™ (Cat#BIO-21046)

The polymerase Chain Reaction was carried out in an automated thermal cycler (PTC-200 M.J Research Inc) according to the respective programme as described below.

Programme PCR55

This programme is used for the amplification of partial G gene from cDNA, nested PCR and heminested PCR.

Step 1	Initial denaturation	95 °C	10 minutes
Step 2	Denaturation	93 °C	0.75 minutes
Step 3	Annealing	55 °C	0.75 minutes
Step 4	Elongation	72 °C	0.75 minutes
Step 5	Repeat step 4 for 35 times		
Step 5	Final Elongation	72 °C	0.75 minutes
Step 6	Keep	4 °C	∞
Step 7	End		

Programme PCR50

For glycoprotein gene amplification and colony PCR

Step 1	Initial denaturation	95 °C	10 minutes
Step 2	Denaturation	93 °C	1 minutes
Step 3	Annealing	50 °C	1 minutes
Step 4	Elongation	72 °C	1 minutes
Step 5	Repeat step 4 for 35 times		
Step 5	Final Elongation	72 °C	1 minutes
Step 6	Keep	4 °C	∞
Step 7	End		

3.15.5 **Plasmids**

pSC11 plasmid (Chakrabarti *et al.*, 1985; Chiam *et al.*, 2009) was a gift from Dr Rachael Chiam, Cambridge University.

pTM3 was a gift from Dr. Bernard Moss, Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, USA.

3.15.6 **Colony PCR**

Colony PCR was developed for rapid screening of bacterial clones containing the positive inserts in pGEM-T Easy (Cat#A1360) (Promega), pSC11 (Chakrabarti *et al.*, 1985) and pTM3.

A target colony was picked using a 200 µl yellow tip (S1111-0006) (Starlab Group) and resuspended in 100 µl of DEPC water in 0.6 ml microfuge tube, vortex mixed and 10 µl was used as the template for PCR using Programme PCR50.

The PCR products were resolved in agarose gel as described in section 3.15.9.

The remaining bacterial suspension was stored at 4 °C for culture

3.15.7 **Ethidium bromide decontamination**

Used EtBr in TBE buffer was decontaminated through BondEX-50 Ethidium bromide decontamination column, (Cat#740-703), Macherey-Nagel according to the manufacturer's instructions.

3.15.8 **DNA molecular weight markers**

GeneRuler™ 100bp Plus DNA Ladder (Cat#0321) (Fermentas) or GeneRuler™ 1kb DNA Ladder (Cat#SM0311) (Fermentas) was diluted and used according to the manufacturer's instructions.

3.15.9 **Agarose gel electrophoresis**

1 % (w/v) SEAKEM® LE Agarose (Cat#50004) (LONZA) was hydrated for at least 1 minute in 1X TBE solution and solubilised in a 750 W microwave oven at maximum power in bursts of 5 seconds with swirl mixing in between until fully dissolved to a

crystal clear solution. Ethidium bromide was added to the final concentration of 10 µg/ml and poured immediately into a gel cast of Mini-sub® cell (Bio-rad) with the appropriate gel comb and let to solidify at ambient temperature.

The gel together with the gel cast was placed in the electrophoresis cell and submerged in TBE with 10 µg/ml EtBr. 5 parts of DNA was mixed with 1 part of 6X loading dye, loaded into a well and electrophoresed at a contant voltage of 120 V using a power pack (Northumbria Biologicals Ltd) until the desired resolution was achieved.

3.15.10 **Gel purification**

Following resolution by gel electrophoresis, the resolved DNA band to be extracted was excised from the Agarose gel using sterile surgical scalpel blade No.22 (Cat#0308) (Swann-Morton® Ltd) and extracted using QIAGEN Gel Extraction Kit (Cat#28706) according to the manufacturer's instructions. DNA was eluted in 2x15 µl of EB buffer (10 mM Tris-Cl, pH 8.5 elution buffer supplied with the kit) and stored at -20 °C until use.

3.15.11 **Column purification**

Column purification was performed using the QIAquick Gel extraction kit with modification (refer section 3.15.10). Three buffers namely Buffer QG, PE and EB were supplied with the kit. Firstly, 1 part (100 µl) of DNA combined with 3 parts (300 µl) of buffer QG and 1 part (100 µl) of isopropanol. The mixture was passed through the spin column by centrifugation at 13000 rpm for 1 minute and the flow through decanted and the column was incubated for 5 minutes at ambient temperature with 750µl of Buffer PE. Buffer PE was removed by centrifugation as described above and washed immediately with the same volume of Buffer PE. The column was combined with another fresh collection tube and centrifuged for 1 minute to dry the column. DNA was eluted in 2x30 µl of Buffer EB (10mM Tris-Cl, pH8.5) into a 1.5 ml microfuge tube with the lid cut off. Purified DNA was hold on ice or stored frozen at -20 °C.

3.15.12 **DNA concentration**

The concentration of DNA was determined using a Nanodrop ND-1000 spectrophotometer (Coleman Technologies Inc.) and analysed using the default parameters in the Nucleic acid measurement module in NanoDrop version 3.0.0.

3.15.13 **Sequencing**

The DNA concentration determined as described in section 3.15.12 and diluted with DEPC water to 50 ng/µl and sequenced using the Sanger dye termination method by GATC Biotech.

3.16 **Nucleotide sequence analysis**

3.16.1 **Electropherograms**

The electropherograms (file with extension .abi) were analysed manually using GENtle version 1.9.4 (http://gentle.magnusmanske.de/).

3.16.2 **Reverse complement**

Reverse compliment of sequences were created online using reverse complement tool Bioinformatics Organization (http://www.bioinformatics.org/sms/rev_comp.html).

3.16.3 **Sequence assembly**

Sequence assembly were done using the CAP3 Sequence Assembly Program (Xiaoqiu Huang and Madan, 1999). Sequences were manually trimed to the desired length in fasta format (file extension .fas) in text files.

3.16.4 **Sequence alignment**

Sequence alignment was done using ClusterW module and alignment file was saved in MEGA format (.MEG extension).

3.16.5 **Phylogenetic tree**

The phylogenetic tree was constructed based on the sequence alignment (section 3.16.4) using the neighbour joining method, maximum likelihood model with the bootstrap value of 1000 and default random seed value of 31332. The phylogenetic tree generated was saved as .MTS file and edited using the same software MEGA4 Software (Tamura *et al.*, 2007). All other parameters not mention here were carried out as defaulted by the software.

3.17 **Bacterial production of recombinant plasmids**

3.17.1 **Escherichia coli**

E. coli strain TG1 [K12, D (*lac-pro*) *sup* E, *thi*, *hsd*, 5/F' traD36, $proA^{+}B^{+}$, $lacI^{q}$, *lac*ZDM15] was provided by Prof Geoffrey Toms, Newcastle University. TG1 was used to prepare chemically competent cells for the transformation of pSC11, pTM3 and their chimeric constructs.

*E. coli strain JM109 [end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17 (r_k^-, m_k^+), *rel*A1, *sup*E44, Δ(*lac-pro*AB), [F´ *tra*D36, *pro*AB, *laq*I^{q}ZΔM15] chemically competent cell was purchased as a ready to use frozen competent cell.

3.17.2 **Preparation of competent E.coli**

The protocol was kindly provided by Dr. Seok-Mui Wang, University of Malaya which was initially optimized for BL21(DE3) and XL1Blue strains of *E. coli*.

The surface of a frozen TG1 stock (section 3.17.1) was scraped with a flame sterilized platinum loop, streaked on an antibiotic free agar plate and cultured overnight at 37 °C as described in section 3.17.13. A single colony was isolated using a sterile 200 µl yellow tip (S1111-0006) (Starlab Group) and inoculated into a 2 ml LB broth in a bijou bottle and grown overnight at 37 °C with vigorous shaking (200 rpm) in an orbital incubator (Weiss-Gallenkamp)

The overnight culture was diluted 1/100 into 60 ml of prewarmed SOB medium in a 250 ml conical flask and grown to an OD600 of 0.6 at 37 °C with vigorous shaking. The flask was removed from the incubator and held on ice for 10 minutes. The culture was transferred into a 50 ml centrifuge tube and spun at 4000rpm for 10 minutes at 4 °C in a pre-cooled MSE *Chilspin 2* bench centrifuge (Fisons).

The pellet was resuspended in 20 ml of ice cold Tfb1 by gentle swirling and incubate on ice for 10 minutes and spun down as above. The pellet was gently resuspended in 4 ml of Tfb2 and dispensed by 200 µl/vial in screw-cap vial, held on ice for 4 hours and frozen by immersion in liquid nitrogen. The frozen competent cells were stored at -80 °C until use.

3.17.3 **Restriction digest of purified products for ligation**

Cfr91 was used for pSC11 and corresponding gene inserts with Cfr91 restriction site engineered.

Purified DNA was digested with 10 units of Cfr9I (Cat#ER0171) in 1X Buffer Cfr9I (10 mM Tris-HCl (pH7.2), 5 mM $MgCl_2$, 200 mM sodium glutamate,0.1mg/ml BSA) supplied in a 50 µl reaction at 37 °C in automated thermal cycler (PTC-200 M.J Research Inc) for 4 hours. The digested vector was column purified and eluted in 50 µl EB buffer.

NcoI/BamHI digestion was carried out for pTM3 and corresponding gene inserts. Double digestion was not carried out due to the incompability of the digestion buffers for the respective enzymes. Thus, the following proceduce was developed and used.

Purified DNA with 10 units of BamHI (Fermentas) in 1X Buffer BamHI supplied. The digestion was done at 37 °C in an automated thermal cycler. BamHI was removed by column purification (section 3.15.10) and eluted in 50 µl of EB buffer. Then the vector was digested with 10 units of NcoI (Fermentas) in 1X Buffer Tango as above followed by gel purification. The purified vector was eluted in 30µl EB buffer and used immediately.

3.17.4 **Dephosphorylation of vector**

This modified protocol was initially provided by Dr. Soren Nielsen, Newcastle University. Dephosphorylation removes -3’ and -5’ phosphate group from the digested vector preventing self-religation which is crucial for cloning experiments involving a single restriction enzyme or sticky end cloning.

The purified vector was dephosphorylated using 1 unit of calf intestine alkaline phosphatase (CIAP), (Cat#EF0341) in 1X reaction buffer supplied (100 mM Tris-HCl pH 7.5 at 37 °C, 100mM $MgCl_2$) at 37 °C for 30 mins followed by denaturation at 85 °C for 15 minutes in an automated thermal cycler in 100 µl reaction volume. Another 1 unit of CIAP was added to the mixture and incubated as above. The CIAP treated vector was gel purified and eluted in 30 µl of EB buffer. The purified vector was hold on ice and used immediately.

3.17.5 **Ligation**

The insert and the vector were ligated together with 1 unit of T4 DNA Ligase (Invitrogen) (Cat# 15224-017) in 1X ligation buffer supplied (50 mM Tris-HCl (pH 7.6) 10 mM $MgCl_2$, 1mM ATP 1 mM DTT, 5 % (w/v) polyethylene glycol-8000 overnight at 4 °C in a refrigerator. A mock ligation consisting of the vector without insert was included as a vector self ligation control or background control. As the amount of DNA was not quantitated, the volume of insert can be increased up to 6 µl to when relatively low insert DNA concentration was used.

3.17.6 **Insert screening with SmaI**

Restriction digest with SmaI is used with pSC11 and pGEM-T Easy clones. SmaI is a neoschizomer of Cfr9I but is more economical for screening purposes.

Plasmid DNA was digested with 10 units of SmaI (Cat#ER0661) in 1X Buffer Tango supplied (33 mM Tris-acetate (pH 7.9), 10 mM Magnesium acetate, 66 mM potassium acetate, 0.1 mg/ml BSA) for 2 hours at 30 °C in an automated thermal cycler and resolved on a gel. No enzyme denaturation step was done as agarose gel electrophoresis effectively removes the function of SmaI.

3.17.7 **Orientation screening with Eco81I**

Chimeric pSC11 containing positive inserts were digested with Eco81I to determine the insertional orientation.

Plasmid DNA was digested with 10 units of Eco81I (Cat#ER0371) in 1X Buffer Tango supplied (33 mM Tris-acetate (pH7.9), 10 mM Magnesium acetate, 66 mM potassium acetate, 0.1 mg/ml BSA) for 16 hours at 37 °C in an automated thermal cycler and resolved on agarose gel as described in section 3.15.9. No enzyme denaturation step was done as agarose gel electrophoresis effectively removes Eco81I.

3.17.8 **TA cloning**

TA cloning was carried out for the amplification of the gene of interest which failed to be cloned directly into pSC11.

Purified RT-PCR DNA was cloned into a pGEM-T Easy system (Cat#A1360) (Promega) according to the manufacturer's instructions.

3.17.9 **Transformation**

The pipette tips used to transfer the ligation mix to the competent cells were prechilled to -20 °C for an hour before use. SOC medium and ampicillin agar plates were prewarmed to 37 °C before use.

A tube of competent cells was thawed in an ice bath and added to 1-5 µl of the plasmid solution (ligation mix or plasmid). Then the cells were held on ice for 1 hour before heat shocked at 42 °C for 45 seconds in a waterbath and transferred to an ice bath. The cells were resuspended in 800 µl of prewarmed SOC medium, incubated at 37 °C and shaken vigorously for an hour. A volume of 200µl of the culture was plated on ampicillin agar plates followed by overnight incubation at 37 °C. Colonies were picked for screening either by restriction enzyme digest or colony PCR.

3.17.10 **Miniprep Plasmid Extraction**

Plasmid minipreps were carried out using Qiaprep Spin Miniprep Kit, QIAGEN (Cat#27106) according to the manufacturer's instructions.

3.17.11 **Maxiprep Plasmid Extraction**

Maxipreps were carried out using Endofree Plasmid MaxiKit, QIAGEN (Cat#12362) with modifications. All buffers used except growth medium and antibiotic were supplied with the kit.

A single colony was picked using a sterile yellow tip and inoculated into 5 ml of LB broth supplemented with 100 µg/ml ampicillin and grown for 8 hours at 37 °C with shaking at 260 rpm.

1 ml of the culture was inoculated into 500 ml of prewarmed LB broth with 100 µg/ml ampicillin in a plugged 2 litre glass conical flask and grown overnight as above. The following day, the culture was chilled on ice with gentle swirling and pelleted by centrifugation at 5000 rpm for 20 minutes at 4 °C in a JA14 rotor in a Beckman J2-21 centrifuge (Beckman Coultier UK Ltd.). The supernatant was removed by decantation and left to drain by leaving the centrifuge pot in the inverted position on a piece of paper towel for 5-10 minutes.

The pellet was dispersed in 10 ml of Buffer P1 supplemented with 100 µg/ml of RNAseA by vigorous pipetting with a 10 ml serological pipette with a bulb attached. 30 ml of Buffer P1 was added and vortexed vigorously to ensure complete dispersal.

40 ml of prewarmed Buffer P2 was added and mixed thoroughly by invertion and incubated at room temperature for 5 minutes. Lysis was stopped with the addition of 40 ml of prechilled Buffer P3 and mixed well by inversion. The mixture was centrifuged in JLA10.5 rotor at 5000 rpm for 15 minutes at 4 °C without brake to separate the precipitate from the solution.

A QIAGEN-tip 500 was equilibrated with 10 ml of Buffer QBT and allowed to empty by gravity flow. The lysate was filtered through the QIAfilter cartridge into QIAGEN-tip 500. The QIAGEN-tip was washed with 2 x 30 ml of Buffer QC and plasmid was eluted with 15 ml of prewarmed (70°C) Buffer QF.

The plasmid was concentrated by isopropanol precipitation by adding 10.5 ml (0.7 volumes) of isopropanol to the eluate, mixed well by inversion and spun at 14 500 rpm for 30 minutes at 4 °C in a JA20 rotor. The outer side of the centrifuge tube was marked to facilitate to location of the precipitate. The isopropanol was removed by aspiration and washed with 5 ml of 70% (v/v) ethanol and spun at 14 500 rpm for 10 minutes at 4 °C in a JA20 rotor. Ethanol was removed by aspiration and the pellet was left to air dry for 30 minutes. The plasmid pellet was dissolved in 200 µl of Buffer EB and stored at -20 °C.

3.17.12 Preparation of frozen bacterial stock

A single colony was picked and grown overnight. 700 µl of bacterial culture was dispensed into a 2 ml screw-capped vial and mixed with 300 µl of 50 % (v/v) of glycerol stock to make a final concentration of 15 % (v/v) and mix by brief vortexing. The mixture was snap-frozen in liquid nitrogen and stored at -20 °C until use.

3.17.13 Revive bacterial culture from frozen bacterial stock

The surface of the frozen glycerol stock were scraped using a flame sterilized platinum-wire bacterial loop and streaked on a prewarmed LB plate (with ampicillin if applicable) and incubate overnight at 37 °C.

3.18 **HRSV**

3.18.1 **Source of viruses**

HRSV strain A2 and 8/60 representing prototype strain for subgroup A and subgroup B respectively were obtained without passage number from Dr. Sarah E Welsh, Newcastle University. The virus was propagated once in one T75 flask of HeLa cell and resuspended in 4 ml of fresh MM2 and stored as 500 µl aliquot in screw capped vials, snap frozen in liquid nitrogen and stored at -80 °C and used as a virus stock.

Specimens in the form of nose swabs (NSs) and/or nasopharyngeal secretions (NPSs) for Winter 2007/2008 and 2008/2009 which were tested positive by immunofluorescence were obtained from the Health Protection Agency, Newcastle upon Tyne.

Volunteers were recruited by research nurse Kerry Pollard. Specimens in the form of NSs and/or NPSs for Winter 2009/2010 were obtained from recruited volunteer infants hospitalized due to HRSV disease.

Table 9 HRSV strains selected for the source of G gene.

HRSV strain	Genotype	Source	Passage	Reference
2567	SAA1	-	P5	(A. McGill *et al.*, 2004b)
4208	GA2	Isolated during the 07/08 epidemic	P2	-
24702	GA3	-	P5	(A. McGill *et al.*, 2004b)
4908	GA5	Isolated during the 07/08 epidemic	P2	-
25173	GA7	-	P5	(A. McGill *et al.*, 2004b)
5608	BA4	Isolated during the 07/08 epidemic	P2	-

3.18.2 **Virus isolation**

The viscous snort in either NS or NPS was homogenized by pipeting using a sterile 1 ml hydrophobic barrier tip before inoculation. When necessary, the volume can be increased by the addition of HBSS.

The growth medium in the cell culture test tube was discarded by aspiration and 100-500 µl of the unfiltered NPS/NS was inoculated directly onto HeLa monolayer. The tubes were placed in a drum and incubated in a rotary incubator for 1 hour at 37 °C. The volume was adjusted to 1 ml with MM2. The tubes were incubated as above and the media was changed every alternate day until 4+ cytophatic effect (CPE) was observed. Concurrently, a mock infection tube was prepared as a negative control which is comparable to the above except that the inoculums was substituted with MM2. The monolayer was scraped into the medium and a specimen slide was prepared for immunofluorescence. The remaining isolate named passage 1 (P1) was snap frozen in liquid nitrogen as approximately 500 µl aliquot in 2 ml vial and stored at -80 °C.

3.18.3 **Virus propagation**

Passage 1 (or higher passage number) virus stock was thawed rapidly in a 37 °C water bath and then held on ice. 100 µl of the virus stock was transferred into a fresh 2 ml vial and diluted to 1 ml with MM2 and used to inoculate HeLa monolayer grown in 4 oz glass bottle followed by 1 hour incubation with rocking every 15 minutes to prevent desiccation. The volume was adjusted to 10ml with MM2 and incubated for 2 days. The progression of the cytopathic effect was observed under the microscope daily and the medium was replaced every alternate day until 4+ CPE can be observed. The scoring of CPE was based on arbitrary 4 scales scoring with 1+ being the lowest and 4+ being the highest. The monolayer was then scraped into the medium and dispensed as 1 ml aliquots in 2 ml vial, snap frozen in liquid nitrogen and stored at -80 °C.

3.19 Modified Vaccinia Ankara

3.19.1 **Viruses**

MVA strain 575, passage 1 was a gift from Dr Simon Bridge, Newcastle University. The virus was propagated in QT35 cells and regarded as passage 2 (P2) without prior plaquing. The stock titer was 6×10^7 pfu/ml. The same stock was used to generate recombinant MVA throughout this thesis.

9NS3V5His, passage 1 (Chiam *et al.*, 2009) was a generous gift from Dr Rachael Chiam, Cambridge University. 9NS3V5His expressed the NS3 protein of African Horse Sickness disease virus with C-terminal V5 epitope and polyhistidine (6xHis) sequence as a fusion protein under the control of vaccinia P7.5 promoter and co-expressing beta-galactosidase (Bgal) under the control of vaccinia P11 promoter was included as a positive control for Xgal staining. 9NS3V5His was propagated once in QT35 cell line as described in 3.19.3 and the titer for the passage 2 virus was 2×10^7 pfu/ml.

MVA-T, a recombinant MVA expressing the bacteriophage λ T7 RNA polymerase under the control of the vaccinia virus P7.5 promoter (B. Moss *et al.*, 1990) was a gift from Prof. Malcolm McCrae, Warwick University. The virus was received without passage number, propagated once in QT35 and the stock virus was regarded as passage 2 virus.

3.19.2 **Cell disruption**

Cell disruption to release virus particles from cells was immersion of virus infected cells in liquid nitrogen followed by rapid thawing of virus stock in 37 °C water bath. This process was done trice followed by sonication at 3x30 seconds burst in a water bath sonicator with 30 seconds interval between each burst.

3.19.3 **Virus propagation**

MVA and other recombinant MVAs stock viruses were prepared in T75 tissue culture flasks of confluent QT35. The virus stock was thawed rapidly in 37 °C waterbath and aliquoted into 100µl/vial aliquots in 2ml vial and one aliquot was then disrupted as described in section 3.19.2 followed by 1:10 dilution in MM2 and inoculated onto a confluent monolayer of QT35 cells followed by incubation for 1 hour with rocking every 15 minutes. Unused aliquots were snapped frozen in liquid nitrogen and stored at -80 °C. The volume of MM2 was adjusted to 10ml and incubated for 48-72 hours until 4+ CPE can be observed. Cells were then scraped into the medium, transferred into a sterile centrifuge tube (either 15 ml or 50 ml) and pelleted by low speed centrifugation at 1000 rpm for 5 minutes. The medium was removed by aspiration and resuspended in 4 ml of fresh MM2, dispensed as 500 µl aliquots in 2 ml screw-capped vial, snap frozen in liquid nitrogen and stored at -80 °C until use.

T7-MVA stock used for transient expression was resuspended in SAF due to the toxicity of antibiotics to the cell line during transfection.

3.19.4 **Virus titration**

The following method is used to estimate the titer of non-Bgal expressing MVA strain 575 and T7-MVA.

100 µl/vial of virus stock was obtained as described in section 3.19.3 and undergo ten-fold serial dilution in ice cold MM2. Each dilutions was dispensed in duplicate at 200 µl/well in 24 well plate (Corning) and two wells were mock infected with the same volume of fresh MM2 followed by 1 hour incubation. The inoculum was removed by aspiration and the monolayer was washed once with 500 µl/well of PBS and replenished with 500 µl/well of MM2 followed by 48 hours incubation. Plaques should be visible at at 28 hours post infection (p.i). The medium was removed by aspiration and the monolayer was fixed with 500 µl/well of 10 % formal saline for 30 minutes, rinsed once with PBS followed by 100 µl/well of crystal violet solution. Plaques were observed and manually counted under a microscope.

The following method is used to estimate the MVA virus titer which co-expresses Bgal under the p11 vaccinia virus promoter.

The inoculation procedure was carried as described above but after the 1 hour incubation, the monolayer was washed once with PBS and removed by aspiration. Residual PBS was removed by tilting the plate at an angle between 30-45 degrees and aspirated using a 200 µl yellow tip attached to P200 Pipetman micropippeter adjusted to the maximum volume capacity. The monolayer was overlayed with 500 µl/well of agarose overlay, solidified at 4 °C for 15 minutes followed by 48 hours incubation or until plaques cn be observed under a microscope. The second overlay was layered with 500 µl/well of Xgal Agarose overlay, solidified as described above followed by overnight incubation. Bgal expressing plaques will be stained blue and can be counted without the need of a microscope.

The dilution with the most significant number of plaques was counted.

Stock virus titer $=5\varphi \times 10^{\lambda}$ pfu/ml; where λ = dilution factor, and φ=number of plaques counted

3.19.5 **Multiplicity of Infection (MOI)**

The MOI can be determined with the following formula.

$$\text{MOI}=\frac{Virus\ titer\ of\ inoculum\ (pfu)}{\sum cell\ (\text{number of}\ cells\ at\ the\ time\ of\ inoculation)}$$

3.19.6 **Recombination of shuttle vector into MVA**

Lipofectin preparation (Part A)

30 µl of Lipofectin (Cat#18292-011) (Invitrogen) was diluted into 100µl of OPTIMEM and incubated for 45-60 minutes at ambient temperature.

DNA preparation (Part B)

5 µg of plasmid DNA was diluted into 100 µl of OPTIMEM and incubated for 30 minutes at ambient temperature.

DNA-Lipofectin complexes I

This formulation is to be used for pSC11 shuttle vector and its derived constructs

Part A and Part B was combined and incubated for 15 minutes at ambient temperature and the volume was adjusted to 2 ml with OPTIMEM and used immediately.

DNA-Lipofectin complexes II

This formulation is to be used for pTM3 shuttle vector and its derived constructs

Part A and Part B was combined and incubated for 15 minutes at ambient temperature and used immediately.

Procedure

CEF cells were grown without antibiotics in T25 tissue culture flask (Corning) until the monolayer reached approximately 70% confluency. The medium was removed by aspiration and the monolayer was inoculated with wild type (w/t) MVA at the MOI of 0.05 in OPTIMEM medium and incubated for 2 hours. The inoculum was removed and washed once with 5 ml of OPTIMEM. The monolayer was transfected with the shuttle vector by the addition of 2 ml of DNA-lipofectin complex I (see above) and incubated

overnight. The following day, 2 ml of antibiotic free GM1 was added to the culture and incubated until 4+ CPE can be observed. The monolayer was scraped into the media and pelleted by low speed centrifugation at 1000 rpm for 5 min. The pellet was resuspended in 500 µl of MM1, labelled as recombination mix and stored as a single aliquot in a 2 ml vial, snap frozen in liquid nitrogen and stored at -80 °C until use.

3.19.7 **Plaque assay**

Serial plaquing was carried out to screen and enrich the recombinant MVA generated in 3.19.6. First round of plaquing was done on confluent QT35 cells grown in 24 well plate (Corning). The recombination mix was thawed in 37 °C waterbath and 100 µl was transferred into a fresh 2 ml vial and disrupted as described in 3.19.2 and subjected to tenfold serial dilution in MM3. Unused recombination mix was snapped frozen in liquid nitrogen and stored at -80 °C. Each dilution was dispensed in replicates of two from 10^{-1} to 10^{-6} at the volume of 200 µl/well. A pSC11 recombinant virus 9NS3V5His (section.3.19.1), was serially diluted in the identical manner and was included as a positive control for Xgal staining. The wells in the last row were mock infected with MM3. The plate was incubated for 2 hours, washed once with PBS and overlayed with 500 µl of Agarose overlay followed by 48-72 hours incubation. Once plaques can be observed through the microscope, the X-gal Agarose overlay was layered, solidified and incubated overnight. Plaques were picked by stabbing a crystal tip attached to a P20 Pipetman (Gilson) with the volume adjusted to 10 µl. The agarose plug together with blue coloured materials were resuspended in 500 µl of MM3, snap frozen in liquid nitrogen, stored at -80 °C or used immediately.

The subsequent rounds of plaquing were done in 6 wells plates (Corning). The agarose plug suspension was disrupted as described in section 3.19.2 and 100 µl was dispensed into a fresh 2 ml vial and undergo fourfold serial dilution in MM3. Unused stock was snapped frozen in liquid nitrogen and stored at -80 °C. The volume of each dilution was adjusted to 500 µl with MM3 and inoculated onto the monolayer in their respective well and incubated for 2 hours with rocking every 15 minutes to prevent dessication. The monolayer in one of the well was mock infected with 500 µl of fresh MM3 as an uninfected cell control. The inoculums were removed by aspiration and the monolayer was washed once with PBS and residual PBS was removed as described above. The agarose overlay procedure was identical to the first round plaquing except that the volume of each agarose overlay was increased to 1500 µl/well to prevent desiccation of

the monolayer. Blue plaques were observed under microscope and whenever possible, only blue plaques without any adjacent unstained plaque were chosen to be picked for the subsequent round of plaquing. The plaquing procedures were repeated 5-7 rounds.

3.19.8 **Transient expression**

HeLa cells were grown in a 24 well plate (Corning) to approximately 90 % confluency in antibiotics free MM3. The medium was removed by aspiration and superinfected with T7MVA at the MOI of 3 and incubated for 30 minutes. Then, the inoculum was removed by aspiration and the monolayer was washed twice with 2×500 µl/well of OPTIMEM. The infected monolayer was then transfected with 200 µl of DNA-lipofectin complex II prepared as described in section 3.19.6 and incubated for 4 hours. The monolayer was treated with 200 µl of antibiotics free GM3 and incubated overnight. The following day, 4+ CPE should be observed due to superinfection. The cells were scraped into the medium using a disposable plastic Pasteur pipette and slides were prepared as described in section 3.10.3.

3.20 **Glycosylation inhibition**

3.20.1 **Stock Tunicamycin**

One vial of containing 1 mg of Tunicamycin (cat#T7765-1MG) was dissolved in 1 ml of sterile DMSO by direct injection using a hypodermic needle through the rubber septum of the vial preventing dust formation. The stock solution was stored at -20 ºC until use.

3.21 **Peptide synthesis**

164-177: peptide AcNH-HFEVFNFVPCSIC-CONH2 was synthesized by Eurogentec, SePop purified and selectively precipitated to remove salt and organic starting materials used in the synthesis process. The average purity was determined by MALDI-TOF Mass Spectrum-Instrument Autoflex1 to be ~65 % and the yield was 2 mg.

172-187: Peptide 172-PCSICSNNPTCWAICK-187 was synthesized by Dr. J Gray, Institute for Molecular Biosciences, Newcastle University and stored frozen as 200 µl aliquot at -20 °C.

The lyophilysed peptides were dissolved in 100 µl of DMSO and volume adjusted to 1 ml in distilled water to make 2 mg/ml stock solution in 5 % DMSO, stored as 100 µl aliquot at -20 °C until use.

3.22 **Decay curve**

A non-linear regression line (refered to herein as decay curve) was fitted with the single, two parameter exponential decay algorithm using the 'Fit Curve' function with extrapolation in Sigmaplot version 11.

3.23 **t test**

Paired t test was carried out in SPSS statistics version 17 under the 'analyze'; 'compare means' tab and $p<0.05$ was considered significant.

Chapter 4 Molecular Epidemiology of HRSV in Newcastle over 3 epidemics

4.1 Introduction

The prevalence of the epidemic strains of HRSV over 3 epidemics in Newcastle upon Tyne is evaluated in this chapter. Work on the HRSV epidemic seasons 2007/2008 and 2008/2009, which were mainly studied to establish a method for virus isolation and genotyping, involved the isolation of HRSV from nasopharyngeal secretions (NPSs) previously positive for HRSV by immunofluorescence staining (IF) carried out by the Health Protection Agency (HPA), Newcastle upon Tyne. Virus isolation also provided a collection of representative HRSV isolates in circulation for the cloning and expression of the G and the F glycoprotein genes described in Chapter 5. Phylogenetic analysis was carried out on gene sequences amplified from virus isolates which will be discussed later in this chapter. Specimens negative for isolation were not further investigated. In 2009/2010, specimens from recruited volunteers mainly in the form of nose swabs (NSs) rather than NPSs were available due to a major change in the routine diagnostic system employed by the HPA which no longer requires the collection of NPSs for IF and virus isolation. In this season, both virus isolates and isolation negative specimens were subjected to nested or hemi-nested RT-PCR for genotyping.

4.2 Isolation and identification of HRSV

In the 2007/2008 epidemic, 20 NPSs were inoculated into HeLa tubes for virus isolation (Section3.18.2). 17 of these produced characteristic CPE as shown in Figure 12. Isolates were harvested and specimen slides were prepared (section 3.10.2) and IF testing was carried out with HRSV Mab pool as described in section 3.10.4 and the representative result is shown in Figure 13. The isolation rate from NPSs was 85%.

In the following epidemics, a total of 29 NPSs were obtained and inoculated into Hela tubes and 18 of 29 showed characteristics CPE of which 16 were positive with HRSV Mab pool by IF giving an HRSV isolation rate of about 60%.

In the 2009/2010 epidemic, both respiratory and blood samples were obtained from a total of 23 patients of which 2 had both NPS and NS taken, 4 and 19 volunteers had only NPS and NS taken respectively. 500µl of each dilution of NPSs and NSs in HBSS was aliquoted into 2ml screw-cap vial, snap frozen and stored at -80 °C and the remainder was put into Hela tubes for isolation. 6 of 6 NPSs yielded CPE and were confirmed HRSV positive by IF yielding a 100% isolation rate from NPSs. However, only 6 of 21 NSs including two isolated from NPSs showed CPE which were also confirmed to be HRSV by IF giving the isolation rate of about 30% from NS. The overall isolation rate for this epidemic was about 43%.

Table 10 Summary of the isolation and identification of HRSV

	2007/2008		2008/2009		2009/2010	
	NPS	NS	NPS	NS	NPS	NS
Total specimens (n)	20	N/A	29	N/A	6	21
CPE	17	N/A	18	N/A	6	6
HRSV Mabpool	17	N/A	16	N/A	6	6

(A) Infected **(B) Uninfected**

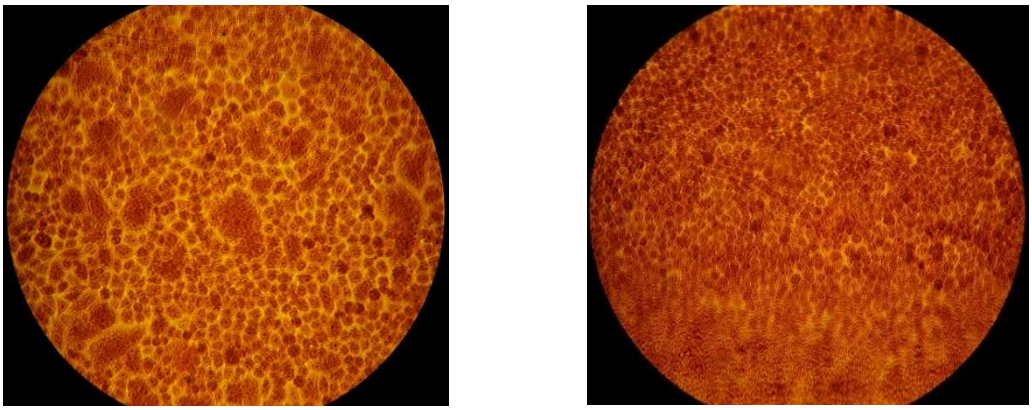


Figure 12 Classical HRSV cytopathic effect

(A) HRSV isolate 5608 passage 2 infected HeLa cells in Hela tube showing classical RSV cytophatic effect compared to (B) uninfected HeLa cells. Cells were fixed and stained using Diff-Quik Stain, Dade Behring Ag, Switzerland and visualized under 100X magnifications

(A) Infected **(B) Uninfected**

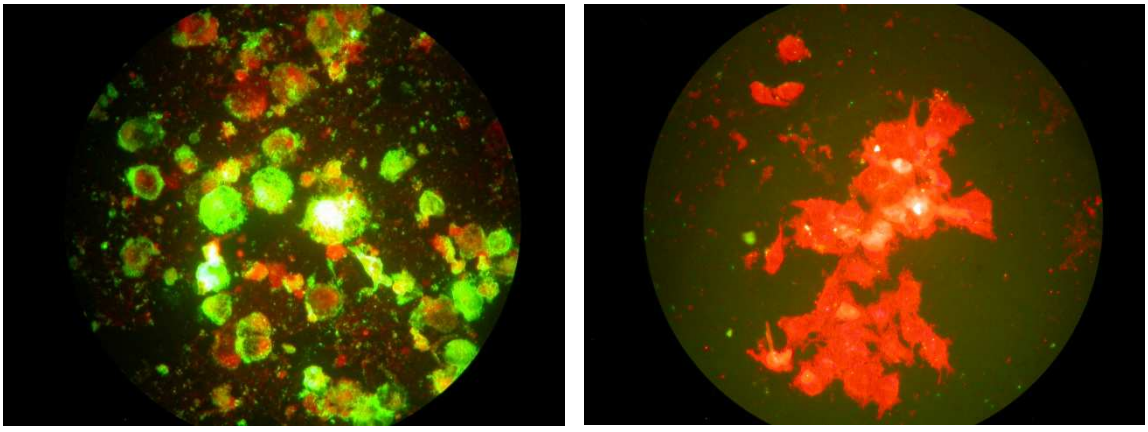


Figure 13 Immunofluorescent staining of HRSV.

(A) HRSV isolate 5608 at passage 2 and (B) uninfected HeLa cells stained HRSV Mab pool; SAM-FITC visualized under 400X magnifications. Apple green staining shows positive fluorescence (A) while red background staining shows negative fluorescence (B).

4.3 Subgrouping of HRSV isolates

During most epidemics of HRSV both virus subgroups and several genotypes co-circulate. Genotyping of HRSV virus isolates is probably best achieved by phylogenetic analysis of the highly variable 3'-terminus of the G gene. In order to achieve this, it was first necessary to establish an RT-PCR system capable of amplifying this fragment.

The confirmed HRSV isolates were passaged in HeLa cells and the total RNA obtained as described in section 3.15.2, and cDNA was synthesized (section 3.15.3) using primer G1 which is universal for both subgroups A and B HRSV. Subgrouping was then done by PCR (section 3.15.4, programme PCR55) using subgroup A specific primer set GC1-F164 and subgroup B specific primer set GB1-GB2

4.3.1 $MgCl_2$ concentration optimization for PCR

The optimization of $MgCl_2$ concentration for PCR was done on the cDNA generated from isolate 4208 passage 2. The PCR mastermix was prepared as described in section 3.15.4 with primer set GC1-F164 except the concentration of $MgCl_2$ was adjusted individually ranging from 4mM to 0.5mM. PCR was carried out with programme PCR55 as described in section 3.15.4 and the PCR products were resolved by agarose gel electrophoresis as described in section 3.15.9 visualized under UV illumination. The results are shown in Figure 14. The expected size of the amplication product was 628bp and the band of interest was the most intense at 4mM of $MgCl_2$ followed by 3mM. A faint band was observed at 2mM $MgCl_2$ and no amplification was observed below that concentration. A non-specific band was observed in between 400-500bp in all concentrations except 1.0mM and 0.5mM. The $MgCl_2$ concentration of 4mM determined for primer set GC1-F164 was also found to work satisfactorily for primer set GB1-GB2.

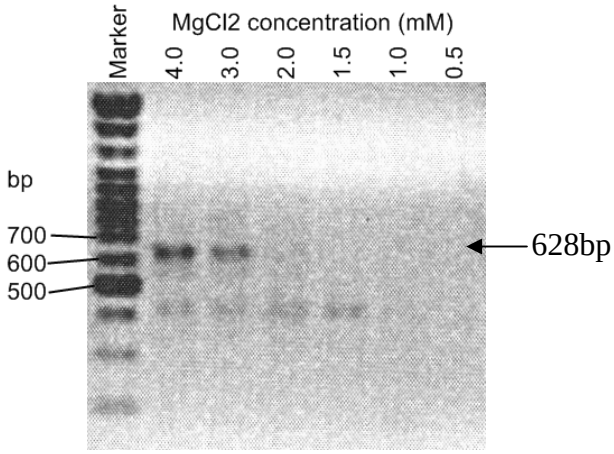


Figure 14 $MgCl_2$ concentration optimization for PCR reaction.

4.3.2 **Amplification of the partial G gene of HRSV isolates**

Representative virus isolates from epidemic 2007/2008 which were confirmed to be HRSV by IF were passaged once in HeLa cells grown in 4oz flat bottom glass bottle to obtain passage 2 viruses (section 3.18.3) followed by total RNA extraction as described in section 3.15.2. The cDNA synthesis was generated as described in section 3.15.3 and PCR was carried out as described in section 3.15.4 with primer set GC1-F164 and resolved on agarose gel as described in 3.15.9. A representative result is shown in Figure 15.

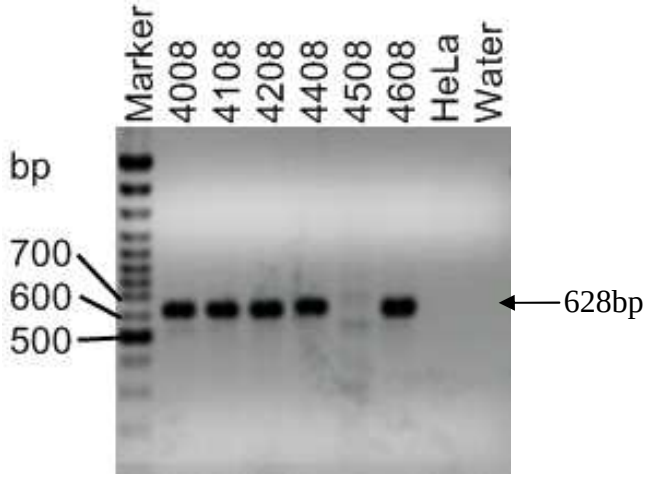


Figure 15 RT-PCR on HRSV isolates using primer GC1-F164

In this example, all isolates yielded a band of the expected size of 628bp except isolate 4508. Non specific bands were not present in the HeLa cell control or water control. Neither A2 nor any other known subgroup A HRSV total RNA extract or cDNA was included as a positive control for reverse transcription and PCR respectively to minimize the possibility of cross-contamination by amplicand from the positive control. cDNA not amplifiable by this primer set was subjected to PCR with GB1-GB2 with programme PCR55 as described in section 3.15.4. and resolved in an agarose gel as described in section 3.15.9. The result obtained with two isolates found to be negative with primer GC1-F164 is shown in Figure 16.

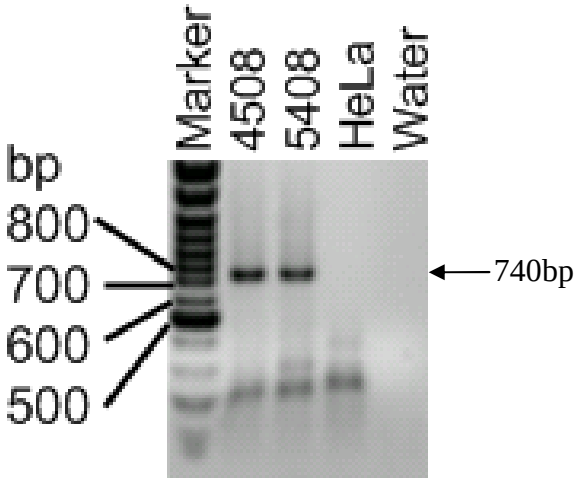


Figure 16 Amplification of the Group B HRSV by using GB1-GB2

Neither isolate yielded a DNA band with the expected size of 680bp as expected from according the genome of HRSV subgroup B prototype strain 9320 but both generated an intense band in between 700-800bp which corresponded to the expected size of 740bp with the HRSV Buenos Aires strain which contains a 60nt repeats in the G gene (Trento *et al.*, 2003). Non-specific band(s) with relatively lower molecular weight than the band of interest are present in both isolates and in the HeLa cell control but not in the water control.

The whole procedure described above was repeated for all the isolates described in section 4.2 and the summary of the results is displayed in Table 11. All cDNAs not amplifiable by GC1-F164 were successfully amplified by GB1-GB2 and yielded band of 740bp.

Table 11 HRSV isolates subgrouping by RT-PCR

Subgroup	Primer set	2007/2008	2008/2009	2009/2010
A	GC1-F164	14	11	3
B	GB1-GB2β	3	5	7
	Total	17	16	10γ

β only PCR bands with the size of 740bp was amplified, γ when HRSV was isolated from both NPS and NS, only the isolate from NPS was used for RT-PCR.

4.4 Subgrouping of HRSV from NSs

The established subgrouping method by RT-PCR from the isolates (section 4.3.2) worked for epidemics 2007/2008 and 2008/2009 without any problem due to the relatively high isolation rate from NPSs. The isolation rate from NPSs remained high in epidemic 2009/2010 but NPSs were only available from 26% (6 of 23) infant volunteers recruited for this study. Although the single round RT-PCR system proved sensitive enough to amplify the partial G gene from RNA extracted from isolates, its sensitivity was insufficient for the RNA extracted directly from the NSs. The genotyping of the specimens is crucial for this study as the maternal antibody titer to the G glycoprotein from the infant volunteers needs to be measured on the recombinant G glycoprotein of the infecting HRSV genotype. Thus hemi-nested and nested PCR strategies were devised for subgroup A and subgroup B HRSV respectively utilizing the available primers and depicted in Figure 17.

4.4.1 **Strategy**

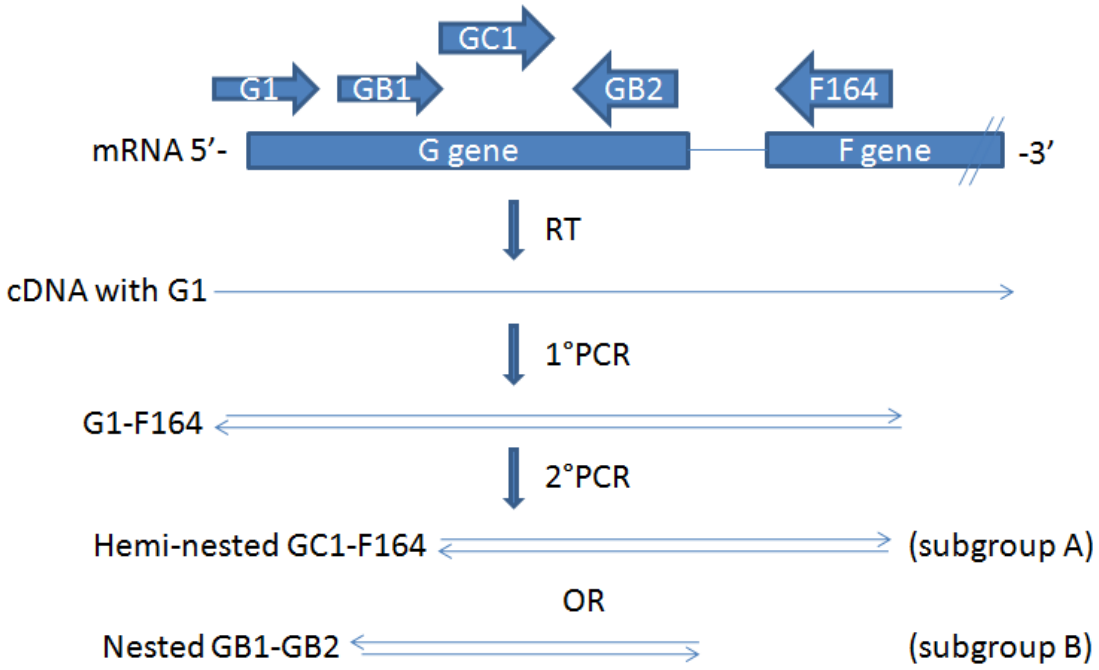


Figure 17 Nested and Heminested PCR strategy.

Diagram not drawn to scale. mRNA is depicted but G1 primer binds to genomic RNA.

The cDNA synthesis was carried out as described in section 3.15.3. The first round PCR (1°PCR) was carried out with primer set G1-F164, which are pan-HRSV primers binding to both subgroup A and subgroup B cDNA, as described in section 3.15.4 using programme PCR55. The second round PCR (2°PCR) was carried out in an identical manner but the PCR product from 1°PCR was diluted 1:1000 in DEPC-H_2O and used as template. The primer sets GC1-F164 and GB1-GB2 was used for the identification of subgroup A and subgroup B respectively.

4.4.2 **Validation**

To test this strategy, two passage 1 isolates, namely 1610 and 4510, representing HRSV group A and B respectively were chosen for the optimization of heminested and nested PCR. These two isolates were chosen for 2 reasons; Firstly, HRSV was successfully isolated, confirmed by IF and genotyped by phylogenetic analysis (Figure 22 and Figure 23). Secondly, for both isolates sufficient NS was available to be used to validate the nested/heminested PCR and also to be included as a positive control in the subsequent assays. The total RNA extracted and cDNA was synthesized as described in section 3.15.3 and the cDNA was serially diluted from 10^{-1} to 10^{-10} in DEPC-H_2O and 10µl of each dilution was subjected to 1°PCR with primer set GC1-F164 as described in section 3.15.4 with the programme PCR55. 10µl of each of the 1°PCR products was resolved in agarose gel as described in section 3.15.9 and the results are shown in Figure 18. Bands of the expected size of 1168bp and 1228bp were observed for 1610 and 4510 respectively. The 1°PCR is sensitive enough to amplify cDNA template of 1610 and 4510 diluted to 10^{-2} and 10^{-6} respectively but this does not indicate differences in the sensitivity of the primer sets as the viral RNAs were not quantitated and standardised for this purpose. Both 1°PCR products were diluted 1:1000 in DEPC-H_20. Diluted templates for 1610 and 4510 were subjected to the 2°PCR with primer set GC1-F164 and GB1-GB2 respectively as described in section 3.15.4 with programme PCR55 and 10µl of the template was resolved in an agarose gel as described in section 3.15.9. The results are displayed in Figure 19. Hemi-nested PCR [Figure 19(a)] on isolate 1610 yielded a band with the expected size of 628bp sensitive down to 10^{-5} which boosted the sensitivity of the 2°PCR up to 10^3 fold compared to 1°PCR. Nested PCR on 4510 did not show any increase in sensitivity compared to the 1°PCR but significantly boosted the intensity of the band of interest [Figure 19(b)]. These strategies proved to be satisfactory in boosting the sensitivity of single round PCR and were used subsequently with the total RNA extracted from the NSs.

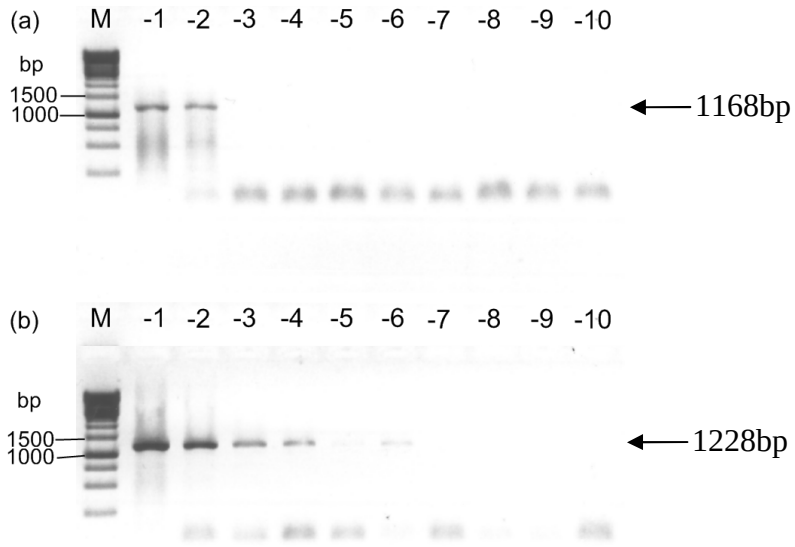


Figure 18 1°PCR amplification.

(a) 1610 P1 (b) 4510 P1 amplified with G1-F164. The negative number indicates the 10-fold dilution factor.

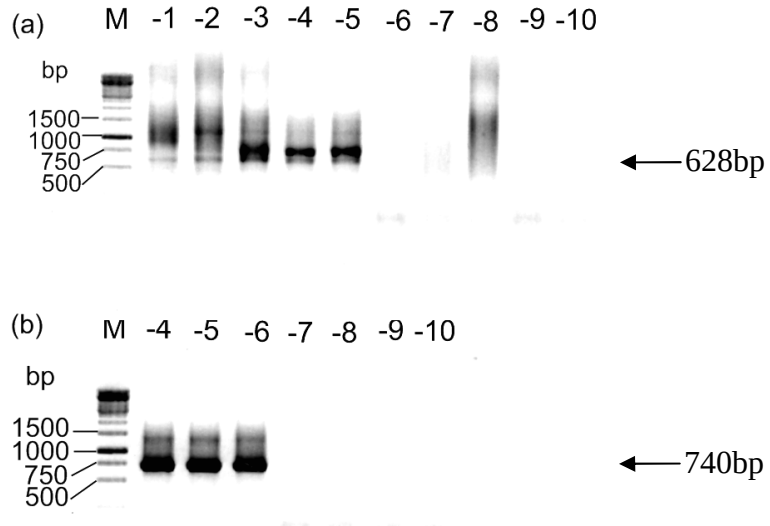


Figure 19 2°PCR amplification.

(a) Hemi-nested PCR on isolate 1610 using GC1-F164. (b) Nested PCR on isolate 4510 using GB1-GB2. The negative number indicates the 10-fold dilution factor.

4.4.3 **Amplification of the partial G gene from NSs**

The total RNA was extracted from the stored NSs (section 4.2) as described in section 3.15.2 and subjected to hemi-nested PCR and nested PCR as described in section 4.4.1 and the results is shown in Figure 20 and Figure 21 respectively.

With reference to the hemi-nested PCR results in Figure 20, specimens 4310, 5110 and the positive control 1610, yielded band with the expected size of 628bp and identified as subgroup A HRSV. Numerous bands were present in which a band with the molecular weight in between 1000-1500bp relative to the marker was present in 1610, 2910, 3210, and 4510 probably represents the product from 1°PCR . Non-specific band with the consistent size between 750-1000bp relative to the marker was present in 2110, 4310, 4610, 4810,5210, 5410. No DNA bands were present in both water controls.

The results of the nested PCR is shown in Figure 20. Two species of dominant band were observed. For subgroup B, or specifically the BA virus, the expected band would be 740bp. This was observed in the positive control for subgroup B, specimen 4510 and also for 2110, 2910, 3210, 4610, 5110, 5210 and 5410 and clearly identified these viruses as subgroup B, BA like. However, a band with the lower molecular weight than the expected size of 740bp was observed in the subgroup A postive control, specimen 1610 and also 4310 and 5010. Without the presence of the subgroup A positive control 1610, it would be tempting to speculate that the lower band corresponded to the molecular weight of 680bp, the expected size of subgroup B prototype strain 9320 non-BA subgroup B. This phenomenon was not observed in the previous epidemics because only non-subgroup A isolates by RT-PCR with GC1-F164 were tested with RT-PCR with GB1-GB2 and the subgroup specificity of primer pair GB1-GB2 was not evaluated. Subsequent sequencing and phylogenetic analysis confirmed that the lower band observed derived from subgroup A HRSV (see Figure 22).

Isolate 2110 yielded a faint band of 628bp with hemi-nested PCR and 740bp with nested PCR. There is a posibility that 2110 is a mixed infection of both subgroups A and B with subgroup B being dominant. However if this is true, nested PCR should amplify 2 bands of 680bp and 740bp corresponding to subgroup A and B respectively but only a single band of 740 was observed in nested PCR thus 2110 is regarded as subgroup B.

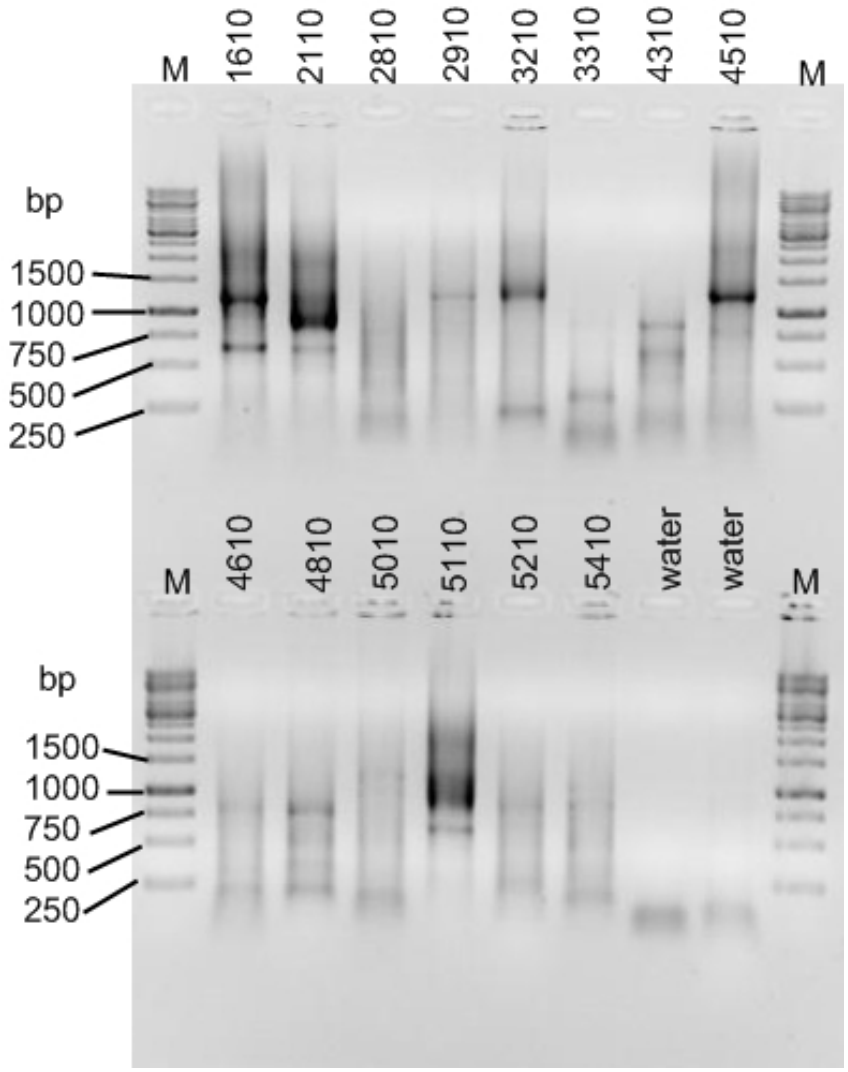


Figure 20 Hemi-nested PCR on total RNA extracted from nose swabs.

The numbers above the bands indicates the specimen number. 1610 and 4510 are considered positive control for Group A and Group B HRSV respectively.

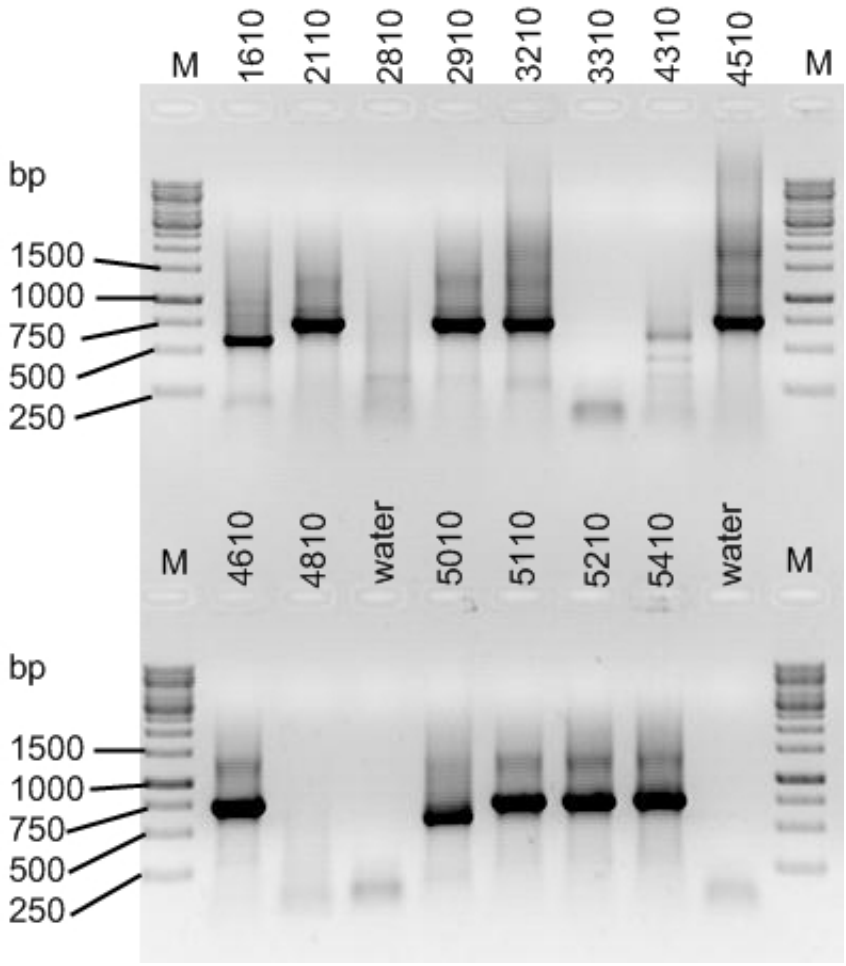


Figure 21 Nested PCR on total RNA extracted from nose swabs.

The numbers above the bands indicates the specimen number. 1610 and 4510 are considered positive control for Group A and Group B HRSV respectively.

4.5 Genotyping by phylogenetic analysis

Sequences from Edinburgh (R), Netherlands (NL) and Bangkok (BK) (Gaunt *et al.*, 2011) were provided by Dr. Eleanor Gaunt, Edinburgh University with sequences from NL and BK initially obtained from Dr. Rogier Jansen, Leiden University Medical Center (LUMC), and Dr. Yong Poovorawan, University of Bangkok.

The PCR products which were positive by RT-PCR described in sections 4.3.2 and 4.4.3 were resolved in agarose gel as described in section 3.15.9 and the appropriate DNA band was excised and gel purified as described in section 3.15.10, quantitated and sequenced as described in section 3.15.13.

The alignment of subgroup A sequences obtained as described above was carried out together with archived isolates from Newcastle upon Tyne (A. McGill *et al.*, 2004a) and representative isolates from Edinburgh (R), Netherlands (NL) and Bangkok (BK) as described in section 3.16. The resulted phylogenetic tree is shown in Figure 22.

The isolation of the BA genotype of HRSV in Newcastle upon Tyne had never been reported until epidemic 2007/2008 due to the absence of continuous epidemiological surveillance thus no archived sequences can be compared with the sequences obtained from the recent epidemics described throughout this chapter. The BA genotypes were the only subgroup B HRSV detected over the three epidemics. To solve this problem, published sequences (Trento *et al.*, 2003; Trento *et al.*, 2010) were included in the alignment and the construction of the phylogenetic tree. No other genotypes other than the prototype CH18537 was included in the analysis and the resulting phylogenetic tree is shown in Figure 23. DNA sequences correspond to nucleotide 526-782 of the G gene of the prototype HRSVstrain CH18537 or nucleotide 526-842 of strain BA/802/99 (Trento *et al.*, 2010).

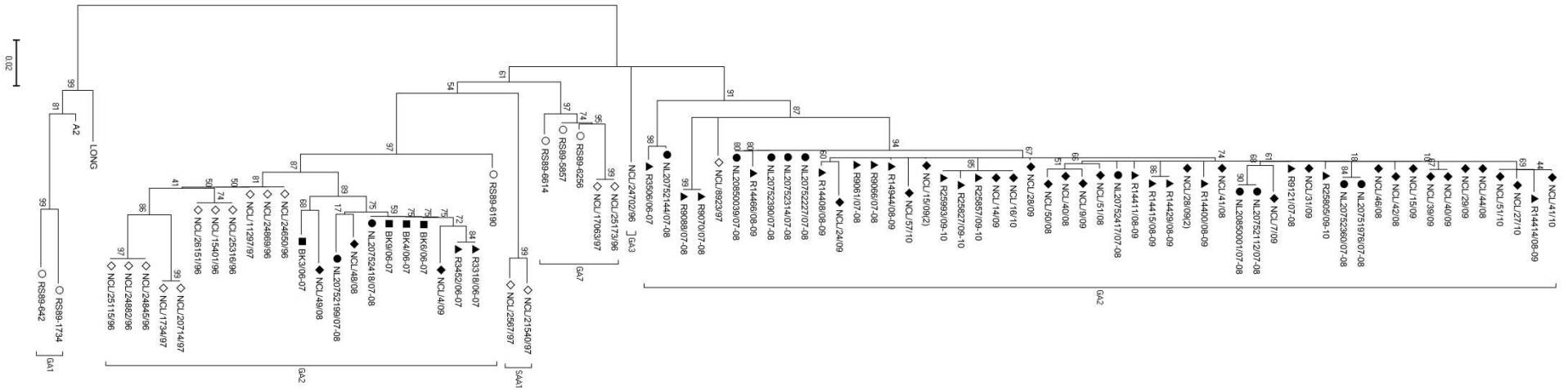


Figure 22 Phylogenetic tree of Group A HRSV.

Partial nucleotide sequences of the second variable region of the attachment G glycoprotein gene of HRSV-A from the 07/08, 08/09 and 09/10 epidemics from Newcastle upon Tyne (◊) (A. McGill *et al.*, 2004b) Newcastle upon Tyne (NCL) (♦) and Birmingham (○) were aligned. Edinburgh (R) (▲), Bangkok (BK) (■) and Neatherlands (NL) (●) isolates. Phylogenetic tree of HRSV-A strains constructed using nucleotide 586-897 corresponding to HRSV strain A2 by the neighbour-joining method, maximum composite likelihood model and bootstrap analysis (1000 replicates) using the MEGA4..

With reference to Figure 22, the recent isolates from Newcastle, Edinburgh and Netherlands can be grouped into a clade identified by the name GA2 according to Peret et al., (2000) and identified with the previously characterized GA2 anchor isolate, NCL/8923/97 (A. McGill *et al.*, 2004a) supported with the bootstrap value of 91. It was interesting to note the following similarity between isolates from different countries: (i) Isolate NCL/27/10, NCL/41/10 and NCL/51/10 sub-clustered together with Edinburgh isolate R14414/08-09 with the bootstrap value of 69. (ii) Isolate NCL/7/09 clustered together with the Dutch isolates NL20752112/07-08 and NL20850001/07-08 with the bootstrap value of 68. (iii) Isolate NCL/14/09 and NCl/16/10 clustered together with R25857/09-10, R25827/09-10 and R25993/09-10 with the bootstrap value of 85. However, all of them were grouped together under a major subclade with the bootstrap value of 94, differing from the ancestral anchor isolate NCL/8923/97 and two recent Edinburgh isolates R9070/07-08 and R9088/07-08. One isolate each from Netherlands; NL20752144/07-08 and Edinburgh; R3506/06-07 subclustered together as an ancestral group to NCL8923/97 which are highly similar with the bootstrap value of 98. No Bangkok isolates was clustered in genotype GA2 clade.

Meanwhile, the other group A Newcastle isolates together with the the rest of the isolates from Netherlands and Bangkok were grouped into a clade named GA5 identified with anchor isolates obtained in 1996,1997 and 1989 shown with the legend ◊ (A. McGill *et al.*, 2004a) supported with the bootstrap value of 97. It was interesting to note that all of the current isolates clustered together as a subclade with the bootstrap value of 89 while the anchor isolates forms a distinct subclade with the bootstrap value of 81 except RS89-6190 (isolated by Patricia Cane in Birmingham) which is considered the veteran genotype for genotype GA5 showing significant evolution of the past from current GA5 genotype.

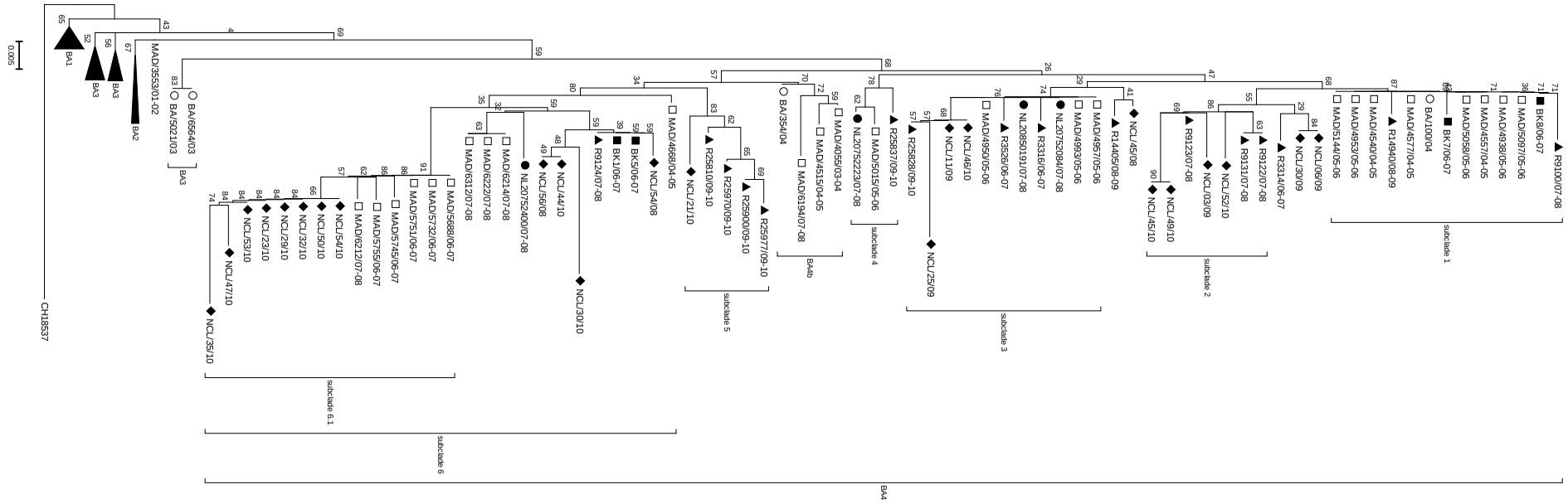


Figure 23 Phylogenetic tree of HRSV BA genotype

Partial nucleotide sequences of the second variable region of the attachment G glycoprotein gene (nucleotides 526-842 of BA/802/99) of HRSV-B from the 07/08, 08/09 and 09/10 epidemics from Newcastle upon Tyne (NCL) (♦)Newcastle upon Tyne (◊)(A. MCGILL *ET AL.*, 2004B) were aligned. Edinburgh (R),(▲), Bangkok (BK) (■),Netherlands (NL)(●) Buenos Aires (BA) (○) and Madrid (MAD).(□). Tree was constructed by the neighbour-joining method, maximum composite likelihood model and bootstrap analysis (1000 replicates) using the MEGA4.Genotypes BA1, BA2 and BA3 was included in the construction of the phylogenetic tree but condensed for better visual.

As Figure 23 shows the Newcastle sub-group B isolates from this study all mapped within the BA4 clade of the phylogenetic tree The BA viruses were novel to Newcastle and were only described in Buenos Aires in 2003. Thus, the anchor sequences for the BA viruses were obtained from published sequences in the genbank (Trento *et al.*, 2006; Trento *et al.*, 2010).

The BA4 viruses were subdivided into 6 subclades. As the interpretation of the bootstrap value, particularly those of less than 70, must be made with care, attribution to sub-clades is uncertain. Subclade1 contains two of each virus from Neatherlands and Bangkok and this clade is supported by a bootstrap value of 87. Subclade 1 viruses cluster together with the MAD-III clade as reported by Trento et al., (2010). Meanwhile subclade 2 contains four members from Newcastle (NCL03/09, 45/10, 49/10, 52/10) and 3 members from Edinburgh (R9122/07-08, R9131/07-08, R9123/07-08) with the bootstrap value of 86. Subclade 3 and 4 correspond to MAD-IV clade which was supported by the bootstrap value of 74 and 78 respectively. Within subclade 3, NCL/11/09, NCL25/09 and NCL46/10 and R2582809-10 were clustered together with a lower bootstrap value of 68 showing their relatedness. Subclade 5 consists of NCL/21/10 and 4 other members from Edinburgh and supported with a stong bootstrap of 83. Subclade 6 contains the highest number of members with the bootstrap value of 80 which includes members from MAD-IV. Within this subgroup, 8 of the Newcastle viruses from epidemic 2009/2010 clustered together with members from the MAD-III clade. The differences in clade assignment between this study and those of Trento et al., (Trento *et al.*, 2003; Trento *et al.*, 2006; 2010) may be due to the different algorithm used in the construction of the phylogenetic tree. The trees in Figure 22 and Figure 23 were constructed using the neighbour joining method but Trento et al., (Trento *et al.*, 2003; Trento *et al.*, 2006; 2010) uses the maximum-likelihood method.

The prevalence of each genotype detected over the three consecutive epidemics in Newcastle upon Tyne is summarized in Table 12. The table shows the gradual decrease in both genotype of subgroup A while the sole representative of subgroup B rose steadily over the study period.

Table 12 Breakdown of prevalent HRSV genotypes in Newcastle upon Tyne over the three epidemics.

Epidemic Year	SubgroupA		Subgroup B
	GA2	GA5	BA4
2007/2008	12	2	3
2008/2009	9	1	5
2009/2010	4	0	13

Chapter 5 Recombinant glycoprotein expression

5.1 Poxvirus expression system

5.1.1 Vaccinia virus

Vaccinia virus belongs to the family of *Poxviridae*, subfamily *Chordopoxvirinae* and genus *orthopoxvirus.* Other members include camelpox, cowpox, ectromelia, monkeypox, raccoonpox, skunkpox, Uasin Gishu, volepox and the notorious variola which causes smallpox (B Moss, 1996).

The origin of vaccinia virus is rather speculative. Edward Jenner himself thought that his cowpox formulation used for the vaccination originated from horses in which the parent virus is now extinct but there is no clear documentation on where or when the modern vaccinia virus emerged. Other hypotheses of the origin of vaccinia virus had been put forward including the hybridization of cowpox and smallpox virus in the early years of vaccination carried out in the Woodville's Smallpox Hospital (Baxby, 1996). However, genomic sequence analysis has revealed distinct differences between the three viruses (M. Mackett and Archard, 1979).

5.1.2 Modified Vaccinia Ankara (MVA)

MVA virus was derived by Anton Mayr by the serial passage of a vaccinia virus derived from a horse through chick embryo fibroblasts more than 570 times, after which it could no longer replicate, or replicated very inefficiently, in a variety of mammalian cell lines. The attenuation of MVA virus has greatly reduced the genome of the virus to 175 kb in length compared to the Copenhagen strain which is 192 kb (Antoine *et al.*, 1998).

5.1.3 Biological safety

Although vaccinia virus is categorised as a class II pathogen (MacNeil *et al.*, 2009), the safety issue of the use of vaccinia virus in research has always been of great concern in the post smallpox era. Vaccinia virus is highly contagious with the need for minimal contact for secondary transmission from vaccinees (Sepkowitz, 2003) or laboratory acquired vaccinia infection (MacNeil *et al.*, 2009).

MVA has a similar host range as its parental vaccinia virus but the host range can be divided into permissive and non-permissive. MVA multiples in permissive hosts such as avian cells, chick embryo fibroblasts (CEF) and the quail cell line (QT35) and the baby hamster kidney cell line (BHK-21). The life cycle of MVA in non-permissive cell lines

is identical to its life cycle in permissive cell lines but the final virion packaging is inhibited (Sutter and Moss, 1992; Sancho *et al.*, 2002).

MVA has consistently passed phase I clinical trial as a vaccine vector (Rochlitz *et al.*, 2003; Richard Harrop *et al.*, 2006; Hawkridge *et al.*, 2008; Amato *et al.*, 2009; Currier *et al.*, 2009; Kaufman *et al.*, 2009; Ramanathan *et al.*, 2009; Sander *et al.*, 2009; Whelan *et al.*, 2009; Amato, 2010; Amato *et al.*, 2010; Currier *et al.*, 2010; R. Harrop *et al.*, 2010; Vasan *et al.*, 2010). Furthermore, IMVAMUNE® a third generation smallpox vaccine candidate formulated using MVA-BN®, a strain of MVA which is now under preparation for phase III clinical trial, was found to be well tolerated without any unexpected side effects by 2800 vaccinees including those with HIV, eczema and atopic dermatitis (Jones, 2008; Kennedy and Greenberg, 2009).

The use of MVA over vaccinia virus in expression systems for foreign proteins eliminates the risk of laboratory acquired infection and and time consuming deactivation of vaccinia virus after recombinant protein expression.

5.1.4 **Expression under the control of the vaccinial P7.5 promoter**

The early and late phases of vaccinia virus life cycle refered to the time before and after DNA replication. The P7.5 promoter is an early and late promoter as the 7.5kDa polypeptide promoter is synthesized throughout the life cycle of the virus. This promoter is driven by the specific vaccinia virus RNA polymerase and is not affected by the host's RNA polymerase II. P7.5 has a yield 10-20 fold lower than P11 vaccinia virus promoter but nonetheless, remained the most widely used promoter (Michael Mackett and Smith, 1986).

5.1.5 **pSC11 shuttle vector**

The pSC11 vector is a co-expression vector which allows the cloning of foreign genes adjacent to *E. coli* β-galactosidase gene (βgal) within the thymidine kinase (TK) gene of vaccinia virus. The βgal gene is driven by a P11 late vaccinia virus promoter. MVA virus infected tissue culture cells can be co-transfected with a recombinant pSC11 plasmid allowing recombination to occur between wild type MVA virus and the plasmid in the thymidine kinase gene introducing the recombinant and beta galactosidase genes into the virus. Recombination into the TK locus destroys the capability of the recombinant virus to produce enzyme TK, thus resulting in TK minus

(TK-) phenotype recombinants which are selectable in the presence of 5-bromodeoxyuridine (BudR) and/or visually distinguishable from spontaneous TK mutants by the addition of βgal indicator to detect βgal gene expression (Chakrabarti *et al.*, 1985). The pSC11 vector contains no ATG start codon downstream of the vaccinia virus promoter which is ideal for cloning and expression of complete open reading frames which contains their own translational initiation codon. The topographical map of pSC11 is shown in Figure 24.

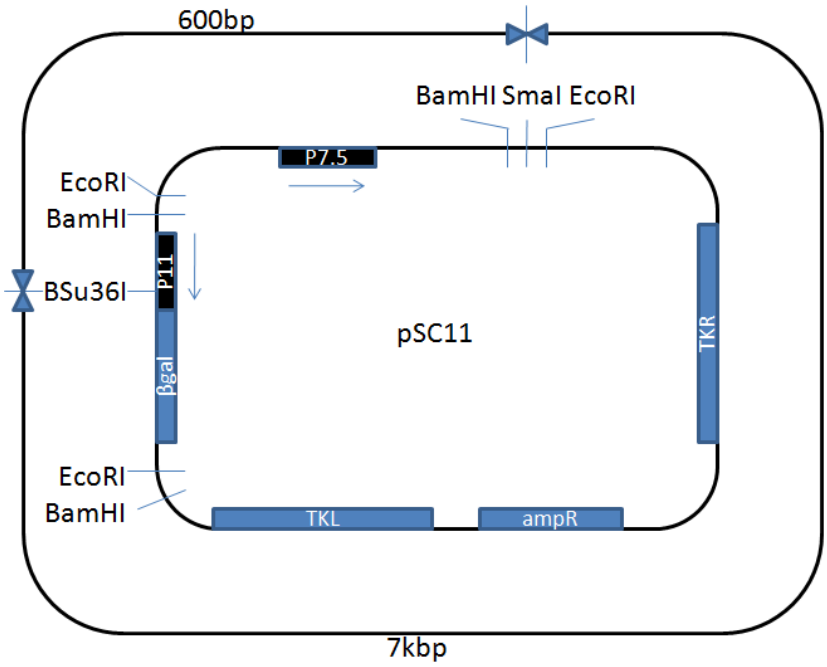


Figure 24 A topographical map of pSC11.

P11, P7.5 are vaccinial promoters; TKR: vaccinial thymidine kinase sequence, right hand; TKL: vaccinial thymidine kinase sequence, left hand; ampR: ampicillin resistance gene; EcoRI, BamHI, Bsu36I, SmaI: restriction sites; SmaI: cloning site for foreign open reading frames.

The cloning of foreign genes into the pSC11 vector is restricted to the SmaI restriction site downstream of the vaccinial P7.5 promoter. The foreign gene does not have to be inserted in frame with the promoter but must carry ATG start codon to initiate transcription and translation. Any non-specific ATG upstream of the actual start codon must be silenced. Kozak consensus sequence (Kozak, 1987) is not required for efficient translation of vaccinia virus mRNA (Michael Mackett and Smith, 1986).

5.2 **Preparation of pSC11 for cloning.**

The insertion of recombinant genes into MVA is facilitated by a shuttle vector such as pSC11 which contains flanking sequence of the thymidine kinase (TK) gene of vaccinia virus where the recombinant gene is inserted. Thus, the very first step was to prepare pSC11 plasmid for the cloning procedures.

pSC11 plasmid prepared from a 1.5ml overnight culture of pSC11/TG1 (section 3.17.10) was digested with Cfr9I (an isoschizomer of XmaI and neoschizomer of SmaI)(section 3.17.3) to produce a sticky end 5'-C/CCGGG-3' and dephosphorylated using CIAP (section 3.17.4). An undigested pSC11 vector was resolved together as an undigested plasmid control as shown in Figure 25. Digested pSC11 migrates as a band between 6k-8kbp corresponding to the expected size of 7.6kbp.

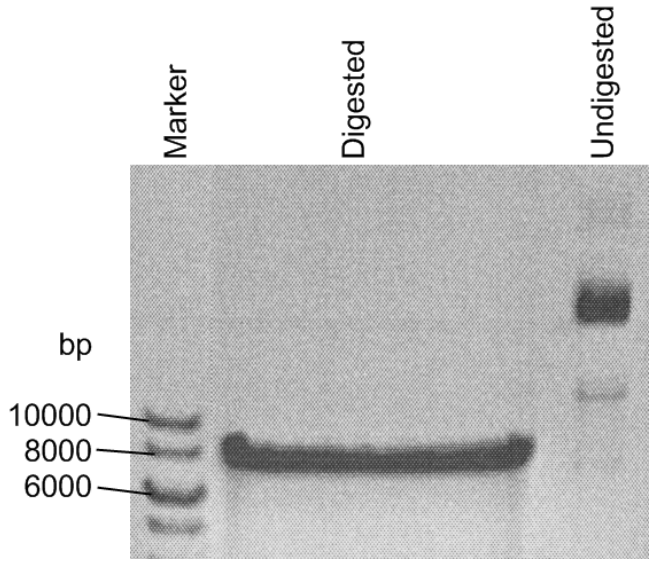


Figure 25 Cfr9I digested pSC11 vector resolved together with native pSC11.

5.3 Preparation of the glycoprotein genes for cloning

As the purpose of this project was to measure maternal antibody titer to the G glycoprotein of the virus genotype infecting infants, the prevalent genotype during the epidemic 2009/2010 could not be predicted and a library of G genes from different genotypes was prepared. A collection of HRSV isolates representing genotype SAA1, GA2, GA3, GA5, GA7 and BA were selected from archived materials and HRSV strains isolated during the 2007/2008 epidemic.

5.3.1 Cloning primer design

All cloning primers are shown in Table 5

The forward cloning primer for the G gene was designed based on strain A2 with a restriction enzyme site Cfr91 added and an ATG codon upstream of the actual start codon silenced to TTG. The mutated adenosine residue is at position -8 from the actual start codon. The primer is named gpGA_For.

The reverse cloning primer for the G gene of subgroup A was designed based on the untrimmed sequence alignment obtained from isolates shown in Table 11 which also provided sequence date used in the construction of the phylogenetic tree as described in section 4.5. This provided sequence data for the end of the G gene, the G/F intergenic region and into the beginning of the fusion gene. Eco86I and Cfr9I sites were included which was named gpGA_Rev. gpGA_Rev binds at the natural stop codon and into the G/F intergenic region which is conserved compared to the -3' end of the G gene. This will allow the use of a single primer to amplify all members of subgroup A and allows each individual strain to use its natural stop codon.

This sequence data was also used for the design of the forward cloning primer for the fusion gene. A Cfr9I restriction enzyme site was added and the primer was named gpFA_For.

The reverse cloning primer for the fusion gene was designed based on the HRSV strain A2 with Eco81I and Cfr9I restriction enzyme restriction enzyme sites added. The primer is named gpFA_Rev.

The forward cloning primer for the BA genotype was similar with gpGA_For but the 5'- end was lengthened to ensure efficient digestion by Cfr9I. To obtain the sequence

data for the design of the reverse primer, PCR was carried out on the cDNA generated from the total RNA of isolate 5608 from section 4.3.2 with primer set G1-F164 as described in section 3.15.4 using programme PCR55. The amplicant was resolved in an agarose gel (section 3.15.9). The DNA band of the expected size of 1228bp was excised and gel purified as described in section 3.15.10 followed by sequencing with primers G1, GB1 and F164 as described in section 3.15.13. The sequence was assembled as described in section 3.16.3. The reverse primer was designed with Eco81I and Cfr9I sites added and named gpGB_Rev.

5.3.2 **Amplification of the G gene.**

The total RNA of subgroup A isolates in Table 9 was extracted as described in section 3.15.2. and the cDNA synthesis was carried out using primer gpGA_For as described in section 3.15.3 followed by PCR using primer set gpGA_For-gpGA-Rev as described in section 3.15.4 using programme PCR50. The DNA was resolved in an agarose gel as described in section 3.15.9 and shown in Figure 26. All five isolates yielded a product with the expected size of 956bp with no significant non-specific band although some faint smear of approximately 500bp was observed in 24702 and to lesser extent in 2567. No non-specific bands were present in either HeLa cell or water control. The primer set was not tested on the A2 strain.

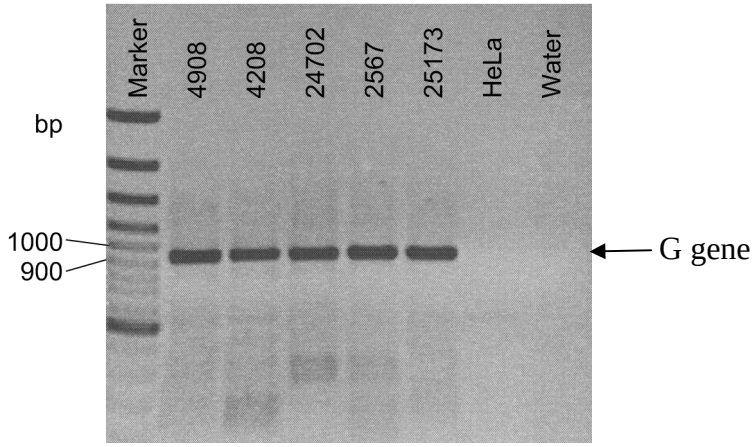


Figure 26 Amplification of the G gene of subgroup A HRSV by RT-PCR

The total RNA of isolates 4508, 5408 and 5608 representing all subgroup B HRSV isolated during the 2007/2008 epidemic was extracted as described in section 3.15.2 and subjected to reverse transcription with primer gpGB-For as described in section 3.15.3 followed by PCR with primer set gpGB_For-gpGB_Rev with programme PCR50 as

described in section 3.15.4. The PCR products were resolved in an agarose gel as described in section 3.15.9 and the results is as shown in Figure 27. All three isolates tested yielded band with the correct size of 1017bp according to strain BA/802/99. Two intense bands of size between 500-750bp and below 250bp were observed in all strains. A faint band with the size in between 750-1000bp was observed but these bands were isolate specific and not due to non-specific binding of primers to the cellular nucleic acids based on the absence of corresponding band in the HeLa cell control. A faint smear was observed in the HeLa cell control but the water control was clean. No further optimization of the annealing temperature was carried out as the amount of DNA in the band of interest was sufficient for the cloning procedure. Only the G gene from isolate 5608 was selected for cloning.

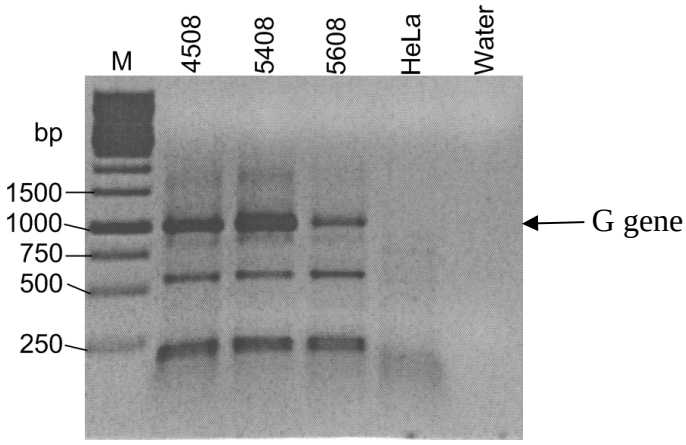


Figure 27 Amplification of the G gene of representative BA4 genotype by RT-PCR.

5.3.3 **Amplification of the F gene**

The RNA extracts from subgroup A isolates used in section 5.3.2 were subjected to reverse transcription with primer gpFA_For as described in section 3.15.3 followed by PCR with primer set gpFA_For-gpFA_Rev using programme PCR50 as described in section 3.15.4. The PCR products were resolved in an agarose gel as described in section 3.15.9 and the result is shown in Figure 28. All isolates tested yielded bands with the expected size of 1796bp based on A2. The water control was clean but two non-specific bands can be observed in the HeLa cell control, both of size in between 750-1000bp.The higher non-specific band was observed in three strains namely 4208, 24702 and 2567 but is most prominent in strain 24702 as is associated with lower intensity of the band of interest. The lower non-specific band was present in all subgroup A strains tested.

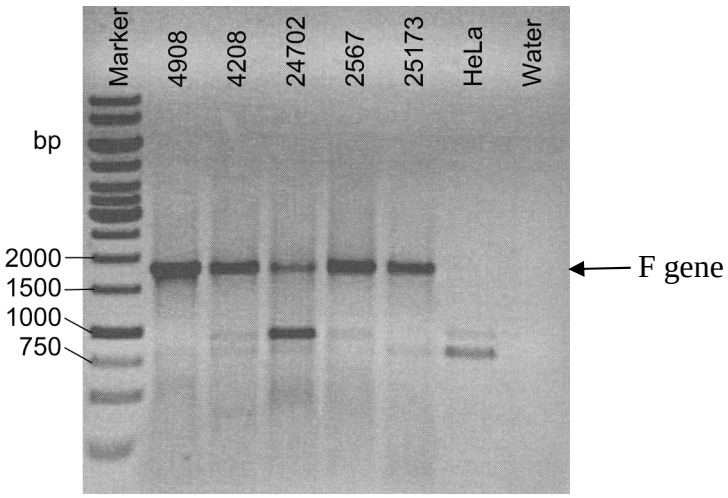


Figure 28 RT-PCR amplifying the HRSV fusion gene of various genotypes.

5.4 Cloning of the inserts into pSC11 vector.

The G genes of subgroup A strain 25173 (GA7), 4208 (GA5) of subgroup A and 5608 of subgroup B, genotype BA4 from section 5.3.2 and the F gene of strain 4208 from section 5.3.3 were digested with Cfr9I as described in section 3.17.3 and ligated into pSC11 as prepared in section 5.1 using the procedures described in section 3.17.5. Isolates 4208 and 5608 were chosen as a representatives for the contemporary genotypes while 25173, a GA7 genotype was chosen as a full length G glycoprotein control in section 7.5.7. The ligated products were transformed as described in section 3.17.9 into chemically competent *E.coli* TG1 as prepared in section 3.17.2. To identify the strain and the gene of each PCR, the strain number will be followed by a suffix G or F representing the G gene and F gene respectively.

All transformations yielded bacterial colonies except for strain 25173G. The procedures above (from RT-PCR on) were repeated thrice without any success probably due to inefficient digestion by Cfr9I. Thus, the PCR product of 25173G was cloned into the pGEM-T Easy vector as described in section 3.17.8 and six colonies was picked and grown in 2ml LB and the plasmids extracted as described in section 3.17.10 followed by insert screening by SmaI as described in section 3.17.6. The result is shown in Figure 29. Three clones were found you harbour the G gene and resolved as a band with the size similar to the PCR product (956bp) namely clone 2, 3 and 5. Clones without the correct insert yielded 2 bands with the size in between 1500-2000bp and 4000-5000bp. pSC11 plasmid was digested with SmaI as a control. Clone 2 of 25173G/pGEM-T, designated as 25173G2 was chosen to be subcloned into pSC11.

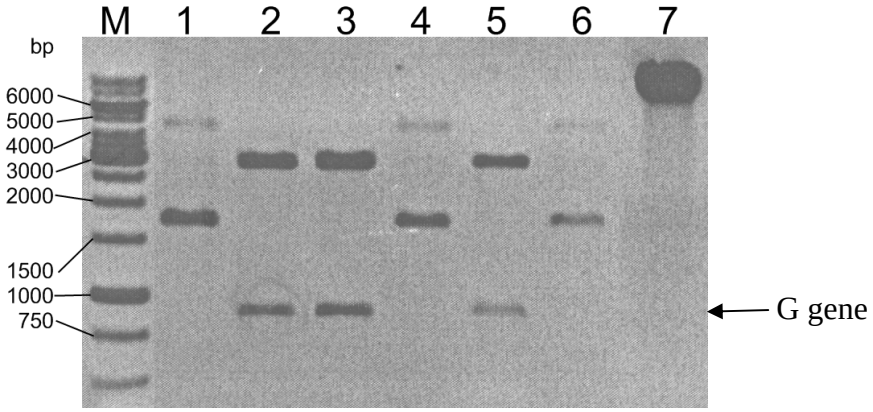


Figure 29 Insert screening of 25173G/pGEM-T Easy by SmaI digestion.

Lane 1-6 represents 6 clones screened, Lane 7: SmaI digested pSC11; M: GeneRuler™ 1kb DNA ladder.

5.4.1 **Insert screening by restriction digest.**

4208G/pSC11 colonies resulting from section 5.4 were picked, grown overnight in LB culture, and plasmid minipreps prepared as described in section 3.17.10. Plasmids were screened by SmaI digestion as described in section 3.17.6 and the result is shown in Figure 30.

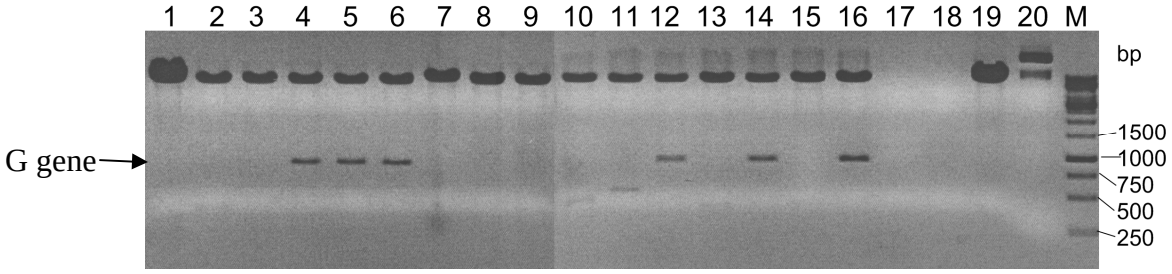


Figure 30 Insert screening of 4208G/pSC11 by SmaI digestion.

Lane 1-18 represents different clones from individual colony. Lane 19:digested pSC11 as a positive control; Lane20; undigested pSC11; M: GeneRuler 1kb DNA ladder

From the 18 colonies picked and screened, 6 colonies were harbouring an insert with the size of about 950bp corresponding to the size of the amplified G gene. They are clone 4, 5, 6, 12, 14, 16. Clones 10 and 11 yielded insert size which did not correspond to the size of the amplified G gene. Clones 17 and 18 contained no plasmid. Glycerol stocks of bacterial clones with insert including those with incorrect size were prepared for storage as described in section 3.17.12. Comparison between the digested pSC11 (lane19) with undigested pSC11 (lane20) shows the complete cleavage of pSC11 by SmaI.

Five resulting colonies of 4208F/pSC11 from section 5.4 were treated as above and the results are shown in Figure 31. Only 1 of 5 colonies screened harbour an insert with a size of about 1790bp corresponding to the amplified fusion glycoprotein gene. Frozen bacterial stock for clone 4208F/pSC11_4 was prepared as described in section 3.17.12.

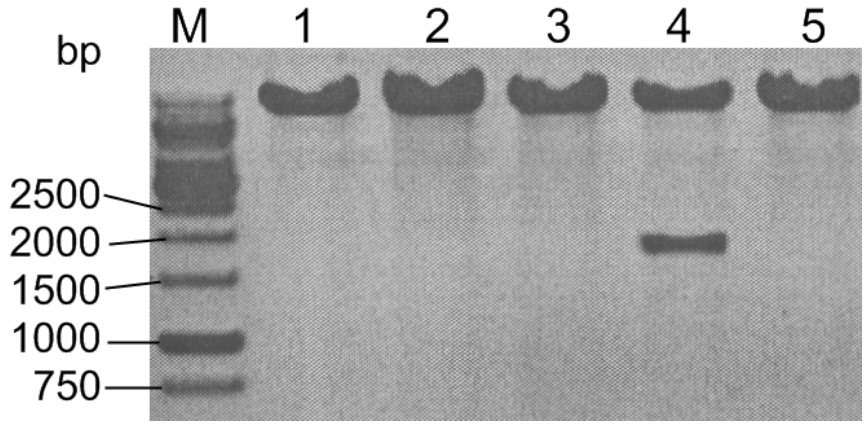


Figure 31 4208F/pSC11 insert screening by SmaI digestion.

Lane number represents actual clone number.

5.5 Colony screening

Colonies was initially screened for inserts by restriction enzyme digest as above. This strategy itself is laborious and lengthy involving overnight growth of bacterial culture, plasmid miniprep, restriction enzyme digestion and agarose gel electrophoresis. Carrying out a large number of plasmid miniprep at any one time can be limited by the capacity of the available centrifuge. Thus, colony PCR was later developed to make colony screening more efficient.

5.5.1 Colony PCR primer set design

In order to design primers for colony PCR, vector sequences flanking the gene insert are required. There is currently no published vector sequence for pSC11. Thus a characterised pSC11/SH2.1 clone containing the SH gene of HMPV was obtained as a frozen bacterial stock from Dr Alison B. Tedcastle, Newcastle University. The original cloning primers used to generate this clone SH Forward and SH Reverse as shown in Table 8.

Both SH Forward and SH Reverse had their sequence upstream of Cfr9I restriction site omitted, reversed complement and the primers were named MCS_upstream and MCS_downstream as shown in Table 7

Miniprep plasmid was prepared from pSC11/SH2.1 as described in 3.17.10, quantitated and sequenced using MCA_upstream and MCS_downstream as described in section 3.15.13. The sequence determined is shown in Figure 32. The sequences were assembled using CAP3 programme as described in section 3.16 and the colony PCR primer set was designed manually and named pSC_For and pSC_Rev.

ACTCAAGACTACGAAACTGATACAATCTCTTATCATGTGGGTAATGTTCTCG
ATGTCGATAGCCATATGCCCGGTAGTTGCGATATACATAAACTGATCACTAA
TTCCAAACCCACCCGCTTTTTATAGTAAGTTTTTCACCCATAAATAATAAAT
ACAATAATTAATTTCTCGTAAAAGTAGAAAATATATTCTAATTTATTGCACG
GTAAGGAAGTAGAATCATAAAGAACA#TCTGTGAGCGTATGGCAACGAAGG
AAAATAGTTATAGTAGCCGCACTCGATGGGACATTTCAACGTAAACCGTTT
AATAATATTTTGAATCTTATTCCATTATCTGAAATGGTGGTAAAACTAACTG
CTGTGTGTATGAAATGCTTTAAGGAGGCTTCCTTTTCTAAACGATTGGGTGA
GGAAACCGAGATAGAGATAATAGGAGGTAATGATATGTATCAATCGGTGTG
TAGAAAGTGTTACATCGACTCATAATATTA

Figure 32 Partial pSC11 vector sequence flanking the MCS derived from pSC11/SH2.1 for colony PCR primer design.

Underlined: binding site for pSC_For and pSC_Rev respectively; # gene sequence not determined.

5.5.2 Validation of colony PCR primer set

In order to validate the newly designed colony PCR primer, a panel of characterised pSC11 clones are required. Thus, 4208G/pSC11 clones 4, 5, 6 (with correct insert size) and clones 10 and 11 (with incorrect insert size) from Section 5.4.1 were chosen to be used in the colony PCR experiment and carried out as described in section 3.15.6. A pSC11/TG1 clone was included as an empty vector control and water for a template free negative control. Results of the colony PCR is shown in Figure 34. The amplification profile of the clones was comparable to the profile of the SmaI restriction digest profile except the corresponding bands were more than 200bp larger than the insert size obtained by restriction digest due to the incorporation of the vector sequence flanking the cloning site. As expected clones 4, 5 and 6 yielded band of similar size above 1000 kb corresponding to the theoretical size of 1191bp and clone 10 and 11 yielded smaller incorrect size bands of approximately 500bp and 600bp respectively.

The colony PCR product generated from empty pSC11 vector was cloned into pGEM-T Easy as decribed in section 3.17.8 and a positive clone was sequenced using the M13 primers set as decribed in section 3.15.13. The sequences were assembled using the CAP3 sequence assembly programme (section 3.16). The sequence is shown in Figure 33 and the length was determined to be 235bp. However, the electromobility size if this fragment on an agarose gel was consistently migrating above 250bp with reference to the GeneRuler 1kb DNA ladder. Sequencing of pSC11 plasmid using pSC_For and pSC_R as described in section 3.15.13 followed by sequence assembly reconfirmed the sequence length to be 235bp.

TTGAAATGTCCCATCGAGTGCGGCTACTATAACTATTTTTCCTTCGTTTGCCA
TACGCTCACAGAATT**CCCGGG**GATCCGTCACTGTTCTTTATGATTCTACTTC
CTTACCGTGCAATAAATTAGAATATATTTTCTACTTTTACGAGAAATTAATT
ATTGTATTTATTATTTATGGGTGAAAAACTTACTATAAAAAGCGGGTGGGTT
TGGAATTAGTGATCAGTTTATGTATA

Figure 33 Sequence of the amplification product generated from native pSC11 vector using the colony PCR primers.

The colony PCR primers pSC_For and pSC_Rev are underlined respectively; The Cfr9I restriction enzyme site is in bold.

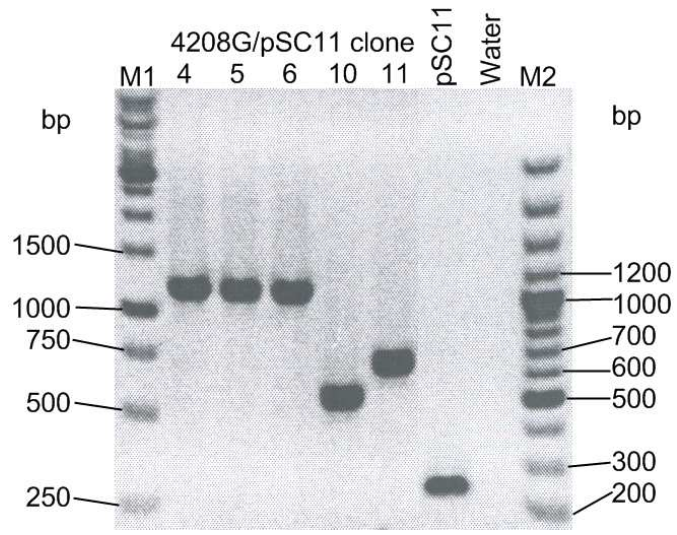


Figure 34 Validation of colony PCR primer for pSC11 using 4208G/pSC11 clones.

Lane number correspond to the actual clone number; pSC11: empty vector control; Water: control without template; M1: GeneRuler™ 1kb DNA ladder; M2: GeneRuler™ 100bp DNA ladder.

5.5.3 **Insert screening by Colony PCR**

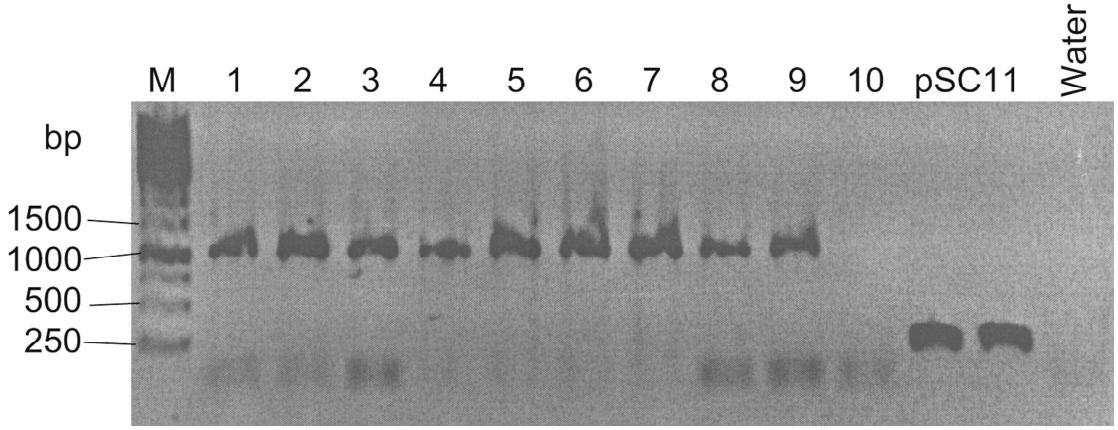


Figure 35 Colony PCR for 25173G2/pSC11.

M: GeneRuler™ 1kb DNA ladder; pSC11 empty cassette template control; Water: template free control.

Ten colonies of 25173G2/pSC11 were picked and subjected to colony PCR as described in section 3.15.6 and the results are displayed in Figure 35. Two pSC11/TG1 colonies were included as an empty cassette control and also as a positive control for the PCR. A water control was included as a template free control or negative control. Clones 1 to 9 yielded a single band of just above 1000bp relative to the marker corresponding to the

expected size of 1191bp and were regarded as positive clones. Clone 10 did not yield any band was probably a satellite colony as negative clones will always give a band at least with the smallest size equivalent to the pSC11 control. Frozen bacterial stocks were prepared for all positive clones as described in section 3.17.12.

Sixteen colonies of 5608G/pSC11 resulting from section 5.4 were picked and treated as described above and the results are displayed in Figure 36. 10/16 5608G/pSC11 clones contain insert of the correct size with the electrophoretic mobility in between 1000-1500bp corresponding to the theoretical size of 1252bp. They are clones 2, 4, 5, 7, 8, 9, 10, 11, 12, and 14. Clone 13 contains no insert but yielded a band with the size equivalent to the band generated by pSC11 vector control. Clones 15 and 16 failed to generate any band probably represent plasmid free satellite colonies. Frozen bacterial stocks were prepared for all positive clones as described in section 3.17.12.

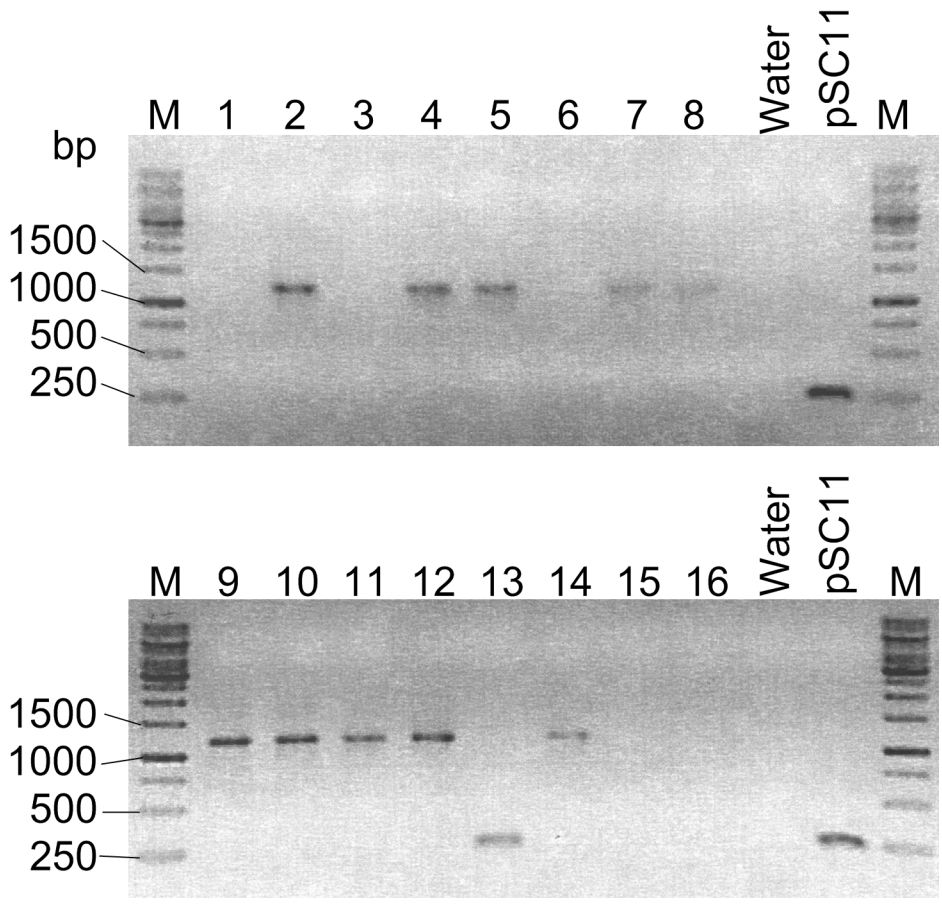


Figure 36 Colony PCR for 5608G/pSC11.

Lane number represents the clone number. Water control and pSC11 plasmid included as controls.

5.6 **Orientation screening**

As the insertion of gene into pSC11 occurs via a non-directional manner through a single restriction site, it can be inserted in either direction. To screen for genes inserted in the correct orientation for translation and transcription, the presence of a unique a second restriction site, Eco81I an isoschizomer of BSu36I site in the P11 promoter of pSC11 was exploited. A second restriction site, Eco81I was engineered into the downstream primer (Table 5) and upon digestion with Eco81I, the enzyme cleaved the two Eco81I sites; one as engineered into the downstream primer and the other located in the p11 vaccinia virus promoter, approximately 600bp upsteam away from cloning site. Thus, plasmid clones with the insert in the correct orientation will yield a band with the size of the insert plus another 600bp from the vector and clones with the wrong orientation will yield a band of approximately 600bp as estimated from Figure 11.

Frozen bacterial stock containing the G gene and F gene prepared in sections 5.5.1 and 5.5.3 were revived as described in section 3.17.13.

The plasmid minipreps of 4208G/pSC11 clones 4, 5, 6, 12, 14 and 16 were digested with Eco81I together with pSC11 plasmid as described in section 3.17.7 and the results are shown in Figure 37. All clones tested yielded a band with the size slightly above 1500bp which corresponded to the size of the G gene insert plus an extra 600bp of vector sequence. Thus all 4208G/pSC11 clones screened here contain the G gene in the correct orientation. 4208G/pSC11_4 was chosen to be recombined into MVA.

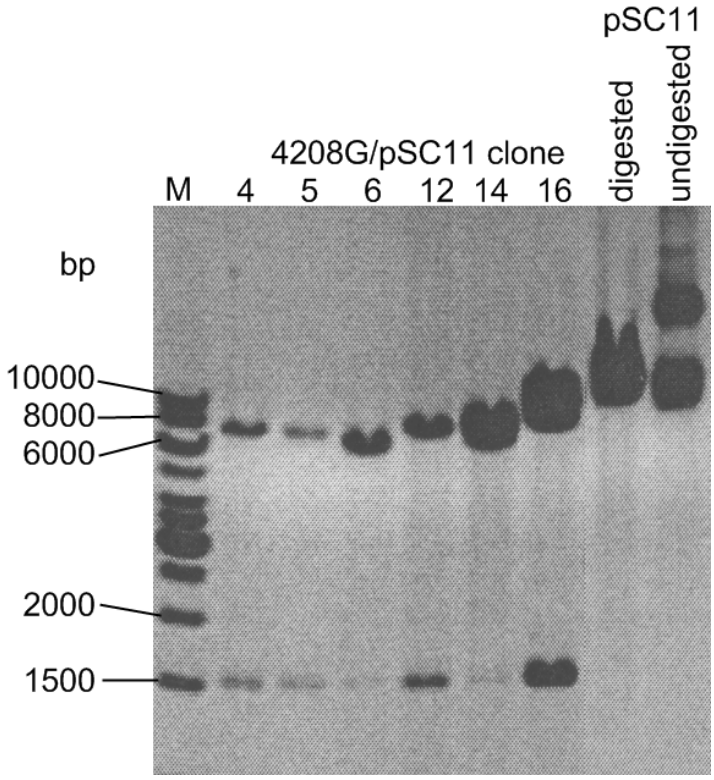


Figure 37 Insert orientation check for 4208G/pSC11 clones by Eco81I digestion.

Numbers on lane correspond to the actual clone number; digested and undigested pSC11 vector for restriction enzyme digestion control.

Plasmid minipreps of 25173G2/pSC11 were treated as above and the result is shown in Figure 38. Clone 1, 4, 6, 7, 8 and 9 yielded a band above 1500bp as described above and are confirmed to contain G gene inserts in the correct orientation. Clones 2, 3 and 5 yielded a band of about 600bp corresponding to the size when an insert in the wrong orientation is present. Thus, clone 25173G2/pSC11_1 was chosen to be recombined into MVA.

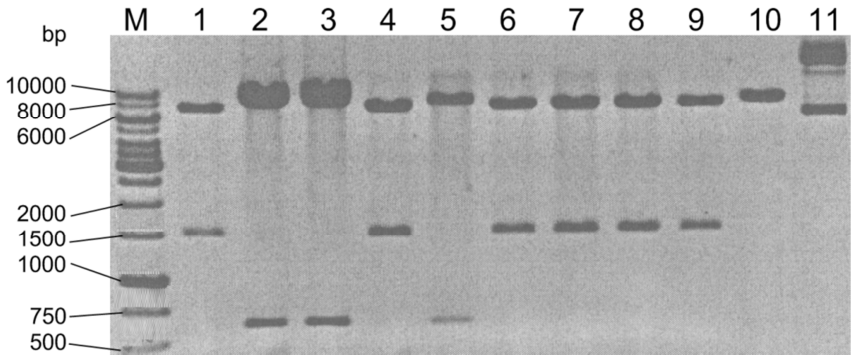


Figure 38 Orientation check for 25173G2/pSC11 by Eco81I restriction enzyme digest.

Lane 1-9 represents individual clones with insert of the correct size; Lane 10: digested pSC11; Lane 11: undigested pSC11.

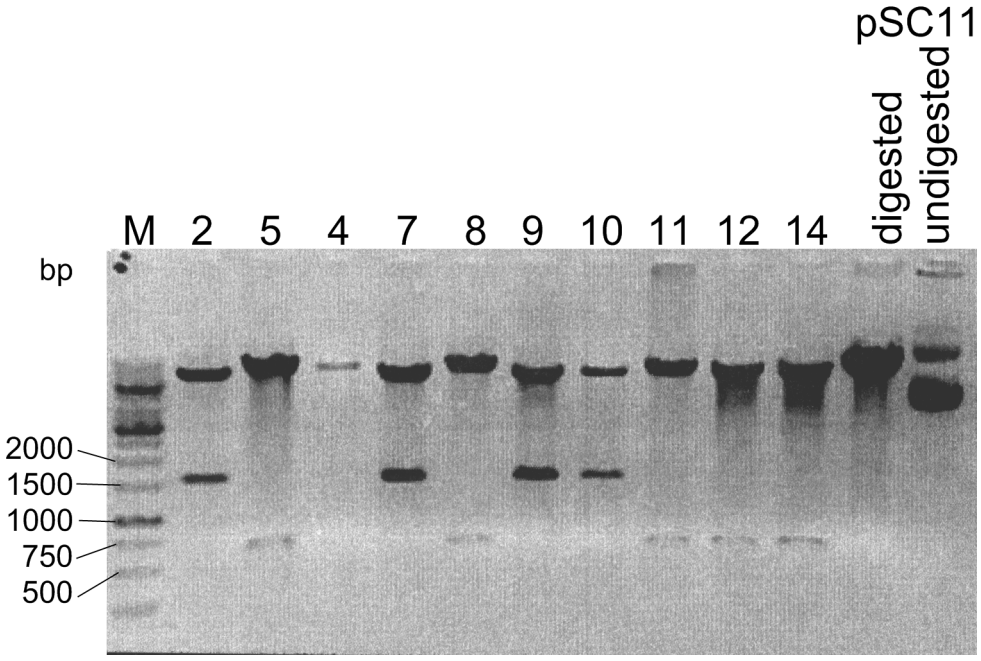


Figure 39 Orientation check for 5608G/pSC11 by restriction digest with Eco81I.

Numbers on lane represents the actual clone number used; digested and undigested pSC11 was included as a control for the restriction enzyme. M: GeneRuler™ 1kb DNA ladder.

Plasmid minipreps from ten positive clones of 5608G/pSC11 were treated as above and the result is displayed in Figure 39. The digestion of these clones with Eco81I shown that clones 5, 7, 9 and 10 contain inserts with the correct orientation yielding a band with the size above 1500bp, which is visually larger than positive sense clones observed in Figure 37 and Figure 38 due to the extra 60 nucleotides insert in the G gene of this BA strain. Clones 5, 8, 11, 12, and 14 contain inserts in the wrong orientation which can be deduced from the lower sized band. The bands from the clones with inserts in the wrong orientation migrate around 750bp relative to the marker, about 150bp higher than the expected size of 600bp. Clone 4 yielded no visible band other than the vector band probably due to the low concentration of the plasmid. Nevertheless, clone 4 was deduced to have the insert in the wrong orientation judging by the larger vector band which is similar to other wrong orientation clones. 5608G/pSC11 clone 2 was chosen to be recombined into MVA.

The orientation screening for pSC11 clones with the fusion gene insert is the same as described above. Clone with the insert in the correct orientation produces a 2.4kbp band while clone with insert in the wrong orientation yielded a 600bp band.

Plasmid minipreps from the sole clone of 4208F/pSC11_4 was digested with Eco81I and the result is shown in Figure 40. The digestion of the clone yielded a band of 2.4kbp showing that this clone contains the F glycoprotein gene inserted in the correct orientation. This clone was chosen to be recombined into MVA.

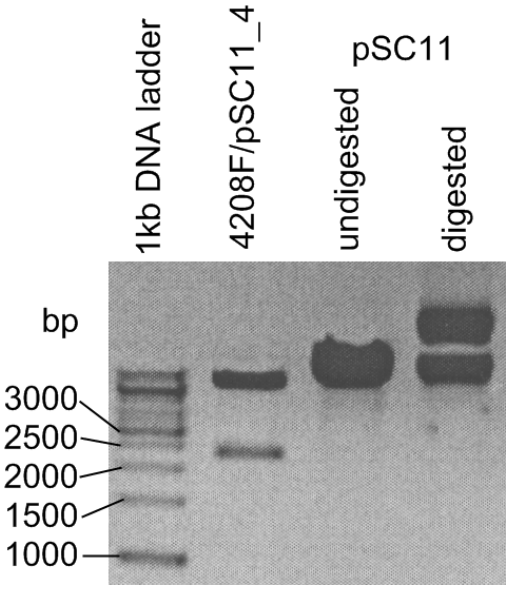


Figure 40 Orientation check for 4208F/pSC11 by Eco81I digestion

5.7 **Plasmid preparation**

High concentration plasmid DNA from selected clones in section 5.6 was prepared by Maxiprep as described in section 3.17.11 and the concentration was determined as described in section 3.15.12 and the results are shown in Table 13. Plasmid 9NS3V5His was used as a positive control for the recombination (section 5.8.3) and recombinant virus enrichment (section 5.8.4) experiments. This provides stocks of plasmid DNA to be used in the recombination experiment described in section 5.8.3.

Table 13 Concentration of recombinant plasmid DNA generated by Maxiprep

Clone	Plasmid	Concentration(ng/µl)
N/A	pSC11	1609.2
9NS3V5His/pSC11	9NS3V5Hisγ	4707.3
4208G/pSC11_4	GA2G	5111.0
25173G2/pSC11_1	GA7G	3204.5
5608G/pSC11_2	BAG	192.8 φ
4208F/pSC11_4	GA2F	3801

φ Clone was not able to grow to high density in large volume, γ (Chiam *et al.*, 2009) The clone was a gift and used as a positive control for the recombination experiment.

Bacterial clone 5608G/pSC11_2 grew normally in 2ml LB but when scaled up to 500ml LB, the bacterium failed to achieve high density and started to lyse judged by the gradual clearing of the culture due to unknown reason.

5.8 The generation of recombinant MVA virus

5.8.1 Cell line selection

A robust cell and sensitive cell line for the replication of MVA was sought. Three permissive cell lines for MVA were evaluated namely BHK21 (section 3.6), CEF (section 3.3) and QT35 (section 3.5). The three cells lines were grown in T75 tissue culture flasks to confluency in their appropriate growth medium as described in section 3.7. The growth medium was removed and the monolayer was infected with 9NS3V5His (section 3.19.1) at the MOI of 0.1 as described in sections 3.19.3 and 3.19.5. Mock infections were carried out in parallel using the appropriate maintenance medium as inoculum. Cultures were incubated and observed daily for CPE and medium replenished every alternate day until 4+CPE can be observed. The cytophatic effect of MVA and its recombinant is identical to the CPE of vaccinia virus which involves the rounding up of cells followed by detachment from the flask.

The virus stocks grown in different cell lines were titered on the parental cell line used to propagate the virus as described in section 3.19.3 The virus titer for 9NS3V5His propagated in both avian cell lines were quite similar with those propagated in CEF, regarded as a gold standard in the propagation of MVA, being the highest. Thus, QT35 and BHK21 produced 0.67 and 2×10^3 times less virus respectively when compared with CEF. Thus, CEF was the most sensitive cell line tested for the propagation of MVA followed by QT35 with lastly BHK21.

Although CEF is the best cell type to be used in the routine MVA procedures such as propagation, plaquing and titering, QT35 was chosen for routine work as it is an immortalized cell line which is more homogenous compared to CEF and can be passaged indefinitely in the laboratory. CEF had been successfully passaged for more than 10 passages but CEF at passage 8, 9 or later tend not to reach full confluency thus restricting its use in the study.

Table 14 Cell line efficiency in cultivating MVA demonstrated using 9NS3V5His, P2

	Number of days for 4+CPE	Virus titer (pfu/ml)
BHK21	3	3.25 x 104
CEF	5	1.50 x 107
QT35	3	1.00 x 107

5.8.2 **MVA stock preparation**

MVA-575 P2 (section 3.19.1), hitherto referred to as MVA, was titrated on QT35 monolayer and plaques were stained with crystal violet as described in section 3.19.4 and the titer was 6×10^7pfu/ml. An example of the stained plaques under 100X magnification is shown in Figure 41.

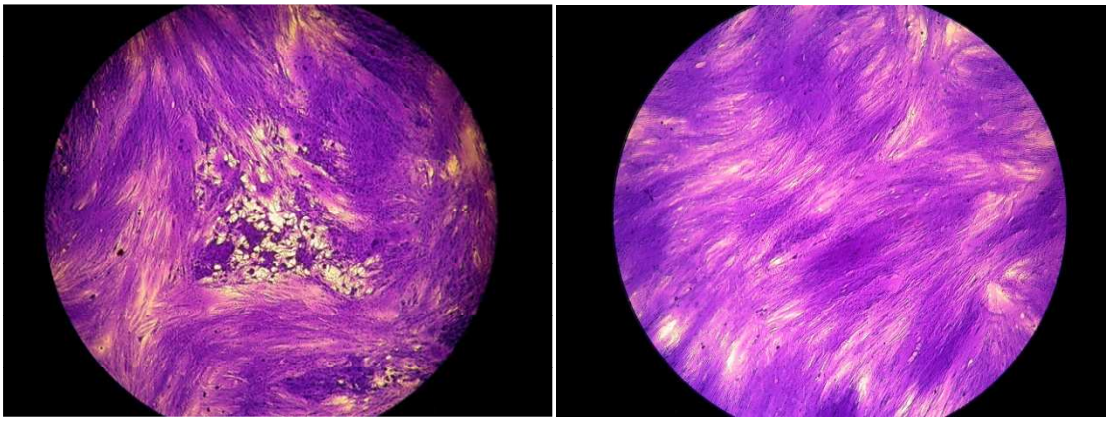


Figure 41 A vaccinia virus plaque and uninfected QT35 cells stained with crystal violet under 100X magnification

5.8.3 **Homologous recombination**

Homologous recombination involves the low frequency recombination of the gene of interest and βgal gene into MVA via cross-overs between the thymidine kinase gene sequences flanking these inserts in the pSC11 vector and the thymidine kinase loci in the virus genome.. The recombinant genome will then be replicated and packaged into progeny virions identified by its TK- phenotype and the ability to express β-

galactosidase due to the presence of the βgal gene driven by the p11 vaccinia virus promoter. Plasmids were diluted to 5µg and recombined into MVA (sections 3.19.1 and 5.8.2) as described in section 3.19.6. The recombination of plasmid 9NS3V5His with MVA was regarded as a positive control as the construct was successfully used in other homologous recombination experiments (Chiam *et al.*, 2009).

5.8.4 **Recombinant virus enrichment**

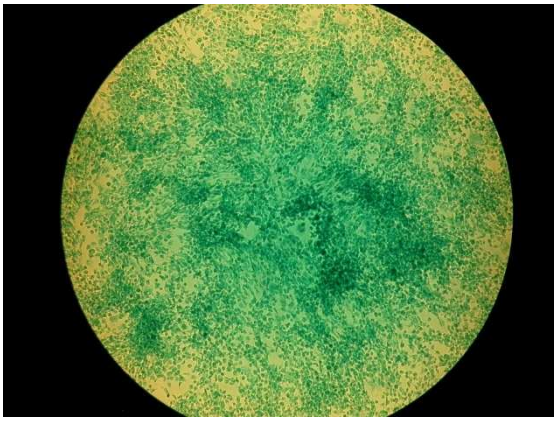
The recombinant MVA viruses can be selected by bromodeoxyuridine (BudR) selection in a thymidine kinase negative (TK-) cell line but this was not available for this study. Instead, the resulting recombinant MVAs expressing the βgal gene were subjected to 5-7 rounds of plaque purification as described in section 3.19.7 with the help of Xgal staining. Recombinant MVA plaques were stained blue by Xgal and could be visualized under the microscope as shown in Figure 42(A) or easily observed by the naked eye, but wild type MVA plaques were colourless and could only be observed through a microscope as shown in Figure 42(B). The choice of blue plaques picked was very selective opting for lone blue plaques without any adjacent wild type or with wild type at a significant distance from the blue plaque to reduce amount of wild type picked together with the recombinant thus producing maximum enrichment of the recombinant virus. Blue foci were confirmed by microscopy to ensure they showed typical poxvirus plaque formation and were not clumps of cells. For unknown reasons, cell clumps exhibited a certain degree of βgal activity and appeared as false positives yielding no viable virus. The plaques from the positive control were not enriched but was solely used as a control for Xgal staining.

Table 15 Recombinant MVAs

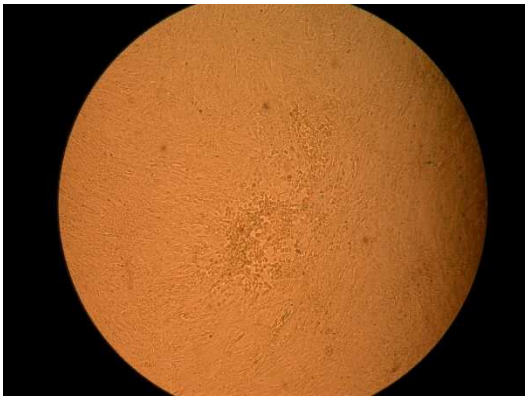
PlasmidΔ	Clone number	Name of recombinant virus	P4 titer
GA2G	1221222	vvGA2G	8.00x107
GA7G	1111313	vvGA7G	5.25x107
BAG	1353632B	vvBAG	1.75x107
GA2F	61393γ	vvGA2F	5.25x107
GA2F	6139311φ	vvGA2F	1.45x107
pSC11	611114	vvBgal	4.25x107

γ clone which produces rapid syncytia in Hela (section5.9), φ clone used in antigen production, Δ nomenclature as defined in

A. Recombinant MVA



B. Wild type MVA



C. Uninfected QT35

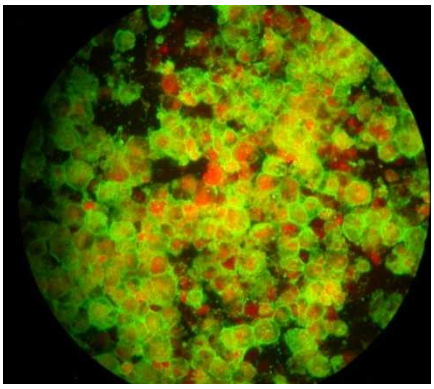
Figure 42 Recombinant and wild type MVA plaques stained with Xgal visualized under 100X magnifications.

5.9 **Expression of G glycoproteins in QT35 and HeLa cells.**

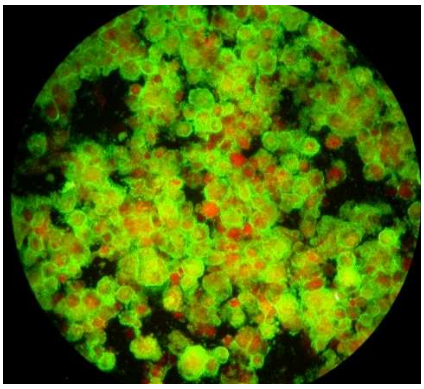
The enriched recombinant MVA expressing the G glycoprotein in section 5.8.4 were propagated on QT35 cells as described in 3.19.3 and fixed specimen slides were prepared as described in section 3.10.3 and stained with the anti-G Mab133 by immunofluorescent as described in section 3.10.4. vvBgal and mock infected QT35 cells were included as controls. The immunofluorescent staining result is displayed in section Figure 43. The G glycoprotein expressed by vvGA2G, vvGA7G and vvBAG were stained apple green as shown in Figure 43 showing the presence of the G glycoprotein in the vvGA2G, vvGA7G and vvBAG infected cells. vvBgal and mock infected QT35 cells were only stained red by Evan's blue counterstain showing the absence of G glycoprotein and the specificity of Mab133 on the G glycoprotein without any observable cross-reactivity on MVA viral proteins, beta-galactosidase nor the cellular proteins.

Recombinant MVAs expressing the recombinant G glycoproteins were titered and inoculated onto HeLa monolayers grown in T75 at the MOI of 3. vvBgal was inoculated in parallel at the MOI of 3 and mock infected HeLa cells were included as controls. The inoculums were incubated for 30 minutes and the volume of each tissue culture flask was adjusted to 10 ml with fresh MM2 and incubated overnight and 4+ CPE was observed the following day. The cells were scraped into the medium and fixed onto slides as described in section 3.10.3 and stained with Mab133 by IF as described in section 3.10.4 and the results are shown in Figure 44. Cells inoculated with vvGA2G, vvGA7G and vvBAG were stained apple green by 133 by IF but not vvBgal and mock infected HeLa showing the expression of G glycoprotein in vvGA2G, vvGA7G and vvBAG infected cells.

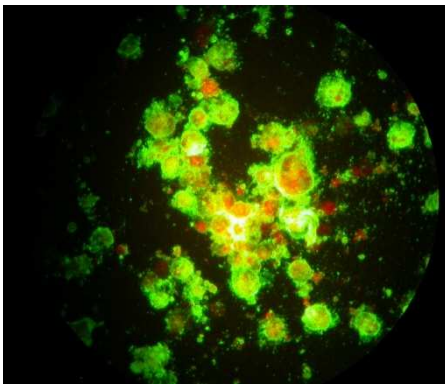
vvGA2G



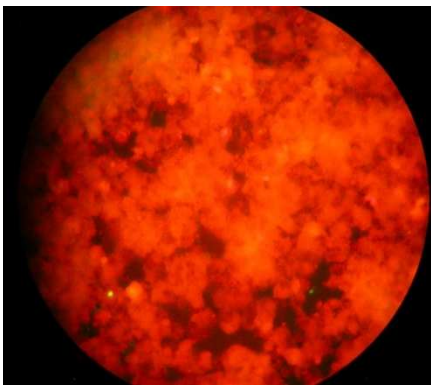
vvGA7G



vvBAG



vvBgal



Mock infected

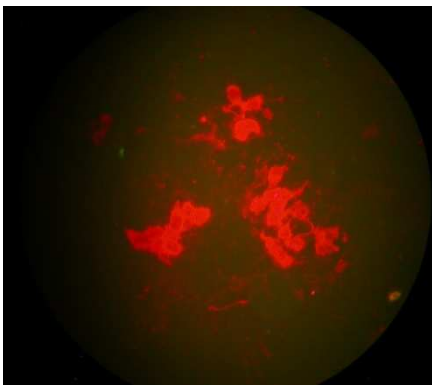
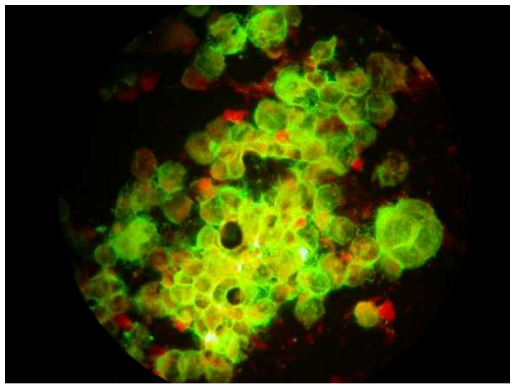


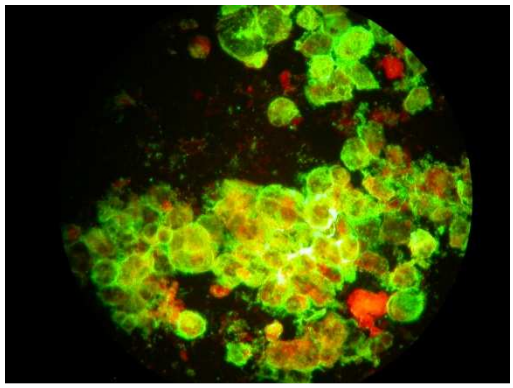
Figure 43 Expression of the recombinant HRSV G gene by recombinant MVAs in QT35 cell line stained with Mab133 by IF and visualized under 400X magnifications.

Apple green staining showed positive IF while red background staining showed negative IF.

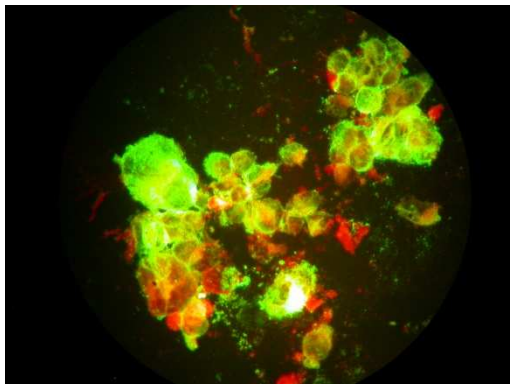
vvGA2G



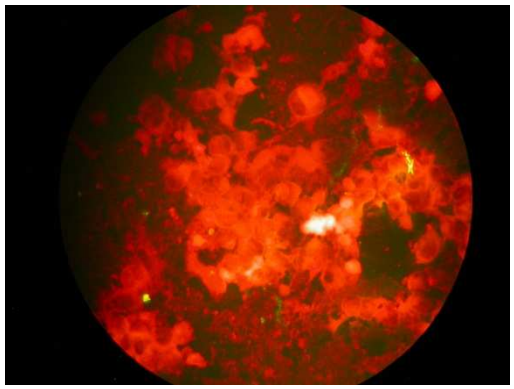
vvGA7G



vvBAG



vvBgal



Mock infected

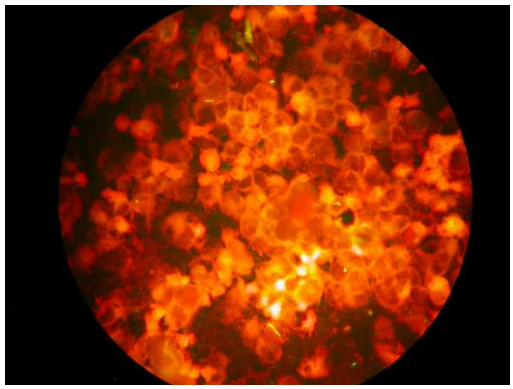


Figure 44 Expression of the recombinant HRSV G gene by recombinant MVAs in HeLa stained with Mab133 by IF and visualized under 400X magnifications.

Apple green staining showed positive IF while red background staining showed negative IF.

5.10 **Expression of F glycoproteins in QT35 and HeLa cells.**

Enriched vvGA2F clone 61393 P4 was inoculated onto QT35 monolayer in T75 as described in section 3.7, absorped for 30 minutes and the volume adjusted to 10ml with fresh MM3. vvBgal and mock infected QT35 was include as controls. The cultures were incubated until 4+ CPE was observed about 48-72 hours post inoculation. No giant cell was observed. The monolayers were scraped into the medium and specimen slides were prepared and fixed onto slides as described in section 3.10.3 and stained with the anti-F Mab 1A12 by IF and the results are shown in Figure 45. vvGA2F infected QT35 cells were stained apple green showing the presence of the fusion glycoprotein. vvBgal and mock infected QT35 were only stained red by Evan's blue counterstain. This showed that the fusion glycoprotein was successfully expressed in by vvGA2F in QT35 and Mab1A12 was specific to the fusion glycoprotein and does not cross react with MVA viral proteins, beta-galactosidase nor the cellular proteins.

vvGA2F vvBgal Mock infected

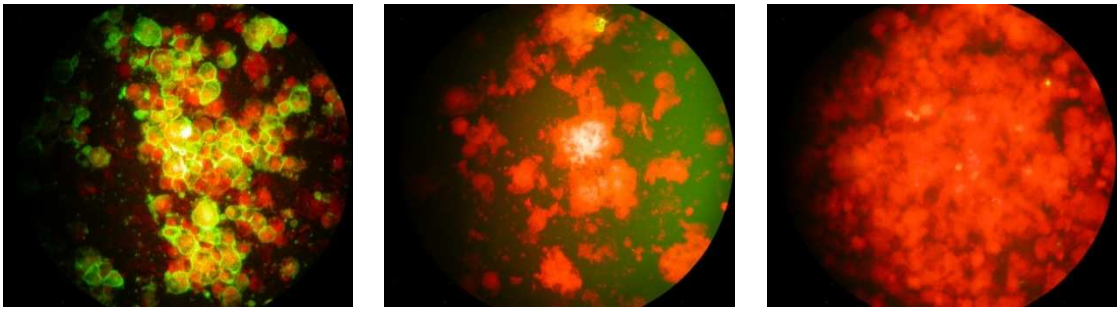


Figure 45 Expression of recombinant fusion glycoprotein in QT35 stained with 1A12.

Apple green staining showed positive IF while red background staining showed negative IF. Slides were visualized under 400X magnifications.

HeLa monolayers were inoculated with vvGA2F clone 61393 P4 at the MOI of 10 and this experiment was repeated with vvBgal. A mock infected HeLa was included as control. The cultures were incubated for 30 minutes and the volume was adjusted to 10ml with fresh MM2 followed by incubation. Upon inspection at 2.5 hours post infection, massive giant cells were observed on vvGA2F infected HeLa monolayer as shown in Figure 46 (Figure 46 was obtained by repeating the experiment and the monolayer was fixed and stained with Diff-quik as described in section 3.8). No formation of giant cells was observed in vvBgal infected HeLa monolayer suggesting

the formation of giant cells were caused by the expression of the recombinant F glycoprotein. 4+CPE was observed the following day with the cells rounding up. The monolayer was scraped into the medium and fixed onto slides as described in section 3.10.3 and stained with Mab1A12 by IF as described in section 3.10.4 and the results are shown in Figure 47. vvGA2F infected HeLa was stained apple green showing the presence of F glycoprotein while vvBgal and mock infected HeLa were stained red with Evan's blue.

vvGA2F vvBgal Mock infected

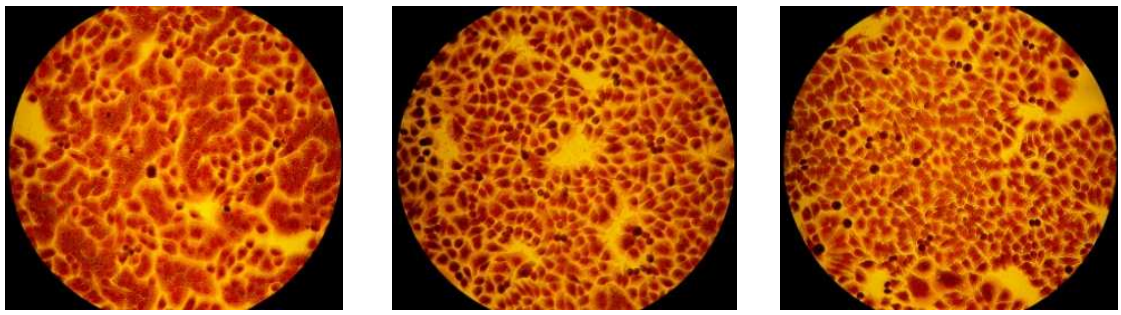


Figure 46 Formation of rapid giant cells by vvGA2F clone 61393 passage 4

Visualized under 100X magnifications

vvGA2F vvBgal Mock infected

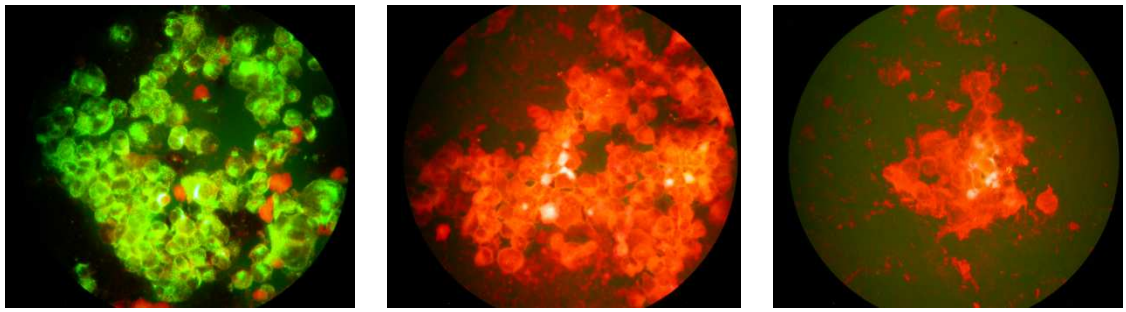


Figure 47 Expression of recombinant fusion glycoprotein in HeLa stained with 1A12.

Apple green staining showed positive IF while red background staining showed negative IF. Slides were visualized under 400X magnifications.

To explore whether synthesis of recombinant F glycoprotein occurred early enough in infected cultures to cause the formation of syncytia at 2.5 hours post-infection vvGA2F

was inoculated onto 6 wells of HeLa monolayer grown in a 24 well plate at the MOI of 10 with vvBgal inoculated in parallel as a control. After 30 minutes of virus absorption, the monolayers were washed trice with 1ml/well of PBS and replenished with 1ml/well of MM2. The monolayers from one set of wells consisting of vvGA2F, vvBgal and mock infected HeLa cells were scraped into the medium and fixed onto individual slides as described in section 3.10.3. These procedures were repeated every 30 minutes until 3 hours post inoculation and fixed specimens were stained with 1A12 by IF as described in section 3.10.4 and the results are shown in Figure 48. The IF staining at t=0.5 hour p.i. showed no visible apple green staining. Apple green staining can be clearly visualized at 1 hour p.i. and the intensity increases over time showing rapid expression of recombinant F glycoprotein in HeLa.

The gradual increase in the fluorescent intensity over 3 hours post inoculation showed rapid synthesis of recombinant F glycoprotein by MVA which is consistant with the rapid formation of syncytia. This suggests that newly synthesized F glycoprotein could be responsible for the rapid formation of syncytia but the possible involvement of F glycoprotein from the inoculums in producing the effect cannot be ruled out. When vvGA2F clone 61393 was serially passaged in QT35, the virus stocks retained the ability to form rapid syncytia when assayed on HeLa monolayers up to passage 7 with later passages losing the ability to form rapid syncytia. Virus titration on higher passage virus stock done as described in section 3.19.4 with Xgal overlay showed the presence of unstained plaques suggesting the presence of wild type MVA. A cloning experiment carried out on the original passage 1 stock as described in section 3.19.7 yielded clone 6139311. Reselection of 6139311 resulted in a wild type free but non-fusogenic virus. The role of the wild type virus in the formation of giant cells is unresolved. It is also possible that 61393 contain a rapid fusion mutant of the recombinant of the recombinant virus but this hypothesis remains to be tested.

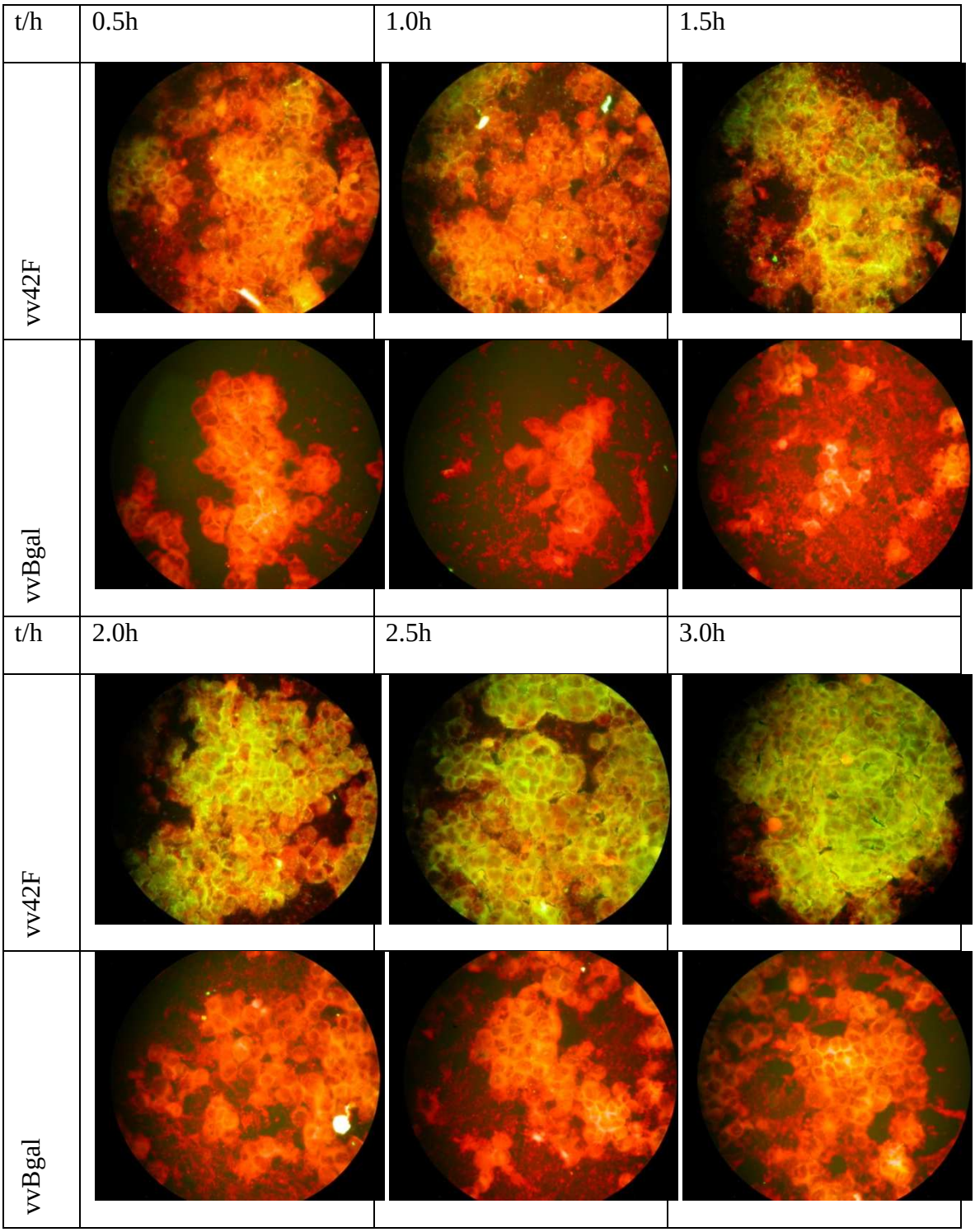


Figure 48 Timeline for the expression of recombinant F glycoprotein at MOI of 10 in HeLa cell line.

vvGA2F clone 61393 P4 vvBgal was used. Cells were stained with Mab1A12 in IF. Apple green staining showed positive IF while red background staining showed negative IF. Slides were visualized under 400X magnifications.

Chapter 6 Development of assays for the measurement of maternal antibodies in infants

6.1 Introduction

This chapter describes the development of a lectin capture ELISA utilizing the recombinant glyproteins described in Chapter 5. These lectin captured recombinant glycoproteins will be used to measure the titer of maternal antibodies to the G glycoprotein of the infecting genotype of HRSV and also to the F glycoprotein of genotype GA2. This chapter will provide a test of the hypothesis that maternal antibody to the genotype specific G glycoprotein is protective.

6.2 Development of concanavalin A capture ELISA

HRSV is known to bind to a series of plant lectins but Concanavalin A (ConA) is probably the most widely used lectin in HRSV research. ConA was previously used in sepharose bead-conjugated forms for the affinity purification of fusion glycoproteins from HRSV infected cell culture lysate (Prince *et al.*, 2000) and recombinant fusion glycoproteins expressed in vaccinia virus and baculovirus expression systems (Wathen *et al.*, 1989a). The same ConA format was also used in the affinity purification of Gs from HRSV infected cell culture supernatant (Teresa R. Johnson *et al.*, 1998; Ray *et al.*, 2001).

ConA capture ELISA was previously developed to capture gp120 of human immunodeficiency virus (HIV) (James E. Robinson *et al.*, 1990) and simian immunodeficiency virus (SIV) (Clements *et al.*, 1995) for the measurement of serum antibodies to the gp120 of the respective viruses. Both authors coated Maxisorp ELISA plates with 2.5µg of ConA in 50µl volume and this amount of ConA was adopted here. ConA was also successfully used to capture recombinant F and G glycoprotein antigens of HRSV expressed from recombinant vaccinia viruses in ELISA (M. J. Robinson, 2007).

6.2.1 **Cell line comparison**

A permissive cell line (QT35) and a non-permissive cell line (HeLa) were chosen to establish the best cell line for the expression of recombinant proteins by recombinant MVA.

Confluent T75 monolayers of QT35 and HeLa cells were inoculated with vvGA2G at a MOI of 0.1 and 3 respectively. Parallel cultures were similarly inoculated with vvBgal. These cultures were treated as described in section 3.19.3 and the antigens processed as described in section 3.11.5 except that, after pelleting, the infected cells were resuspended in 500µl of ice cold SAF.

The concentration of each antigen was determined as described in section 3.14.1 and adjusted to 4mg/ml in SAF. Each was titrated twofold in bicarbonate coating buffer and direct ELISA was carried out in duplicate as described for HRSV antigens in section 3.11.6 with Mab133 (1/100 dilution) and GAMPx as the primary and secondary antibody respectively. Mab133 was diluted 1/100 in PTF, a dilution previously shown to give a corrected optical density of 3.0 with HRSV subgroup B strain 8/60 infected HeLa cell lysate (M. J. Robinson, 2007). Optical densities obtained for antibody binding to the GA2G antigen were corrected by subtraction of binding at equivalent dilutions to Bgal antigen and the results are shown in Figure 49.

The results showed that the GA2G antigen prepared in HeLa cells at high MOI gave higher optical densities compared to that prepared in QT35 cells at a lower MOI. The HeLa cell line was chosen for the expression of all recombinant proteins in MVA for ELISA as use of antigen prepared in human cells may reduce background binding in ELISA of antibodies in human serum.

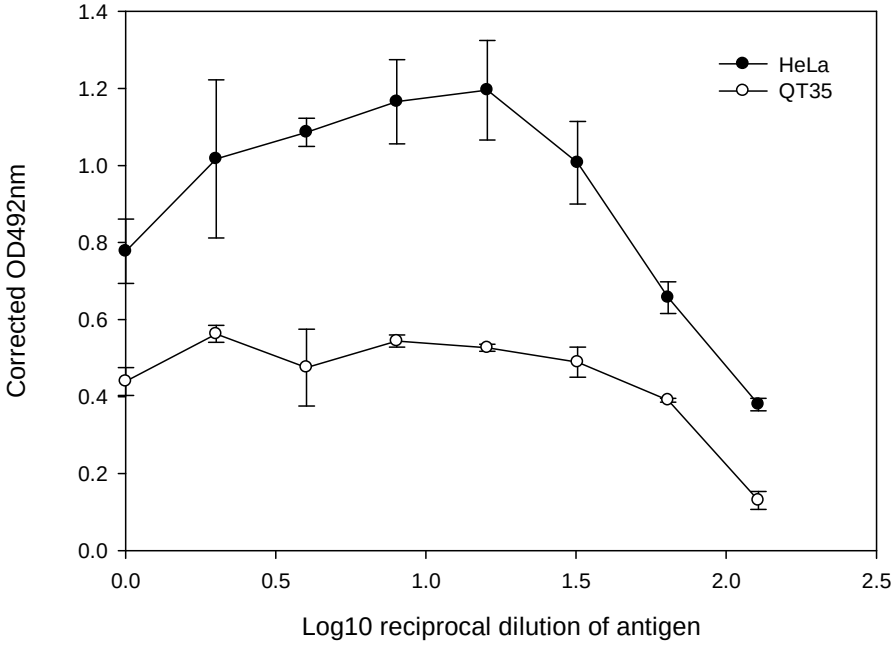


Figure 49 Expression of GA2G in HeLa and QT35 compared in a direct ELISA.

6.2.2 **ConA capture ELISA vs Direct ELISA**

To test if ConA capture ELISA is better than direct ELISA, A comparison was made and is described in this section. Confluent T75 monolayers of HeLa cells were inoculated with vvGA2G at MOI of 3. Parallel cultures were similarly inoculated with vvBgal. These cultures were treated as described in section 3.19.3 and antigens processed as described in section 3.11.5 and the antigens were resuspended in 500µl of ice cold PBSTx.

Half of a Maxisorp plate was coated with ConA diluted in bicarbonate coating buffer and the other half was left blank, incubated overnight and washed as described 3.11.7.

The concentration of recombinant GA2G and Bgal was determined as described in section 3.14.1, adjusted to 4mg/ml in SAF, subjected to twofold serial dilution in PBSTx and dispensed onto the ConA coated wells. In parallel, the antigens were serially diluted in bicarbonate coating buffer and coated on the second half of the plate for direct ELISA as described in section 3.11.6. The plate was incubated for 2 hours and the rest of the procedures were carried out as described in section 3.11.6 with 133 (Table 2) as the primary antibody and GAMPx (Table 3) as the secondary antibody. Optical densities obtained for antibody binding to GA2G antigen were corrected by subtraction

of binding at equivalent dilutions to Bgal antigens and the results are shown in Figure 50.

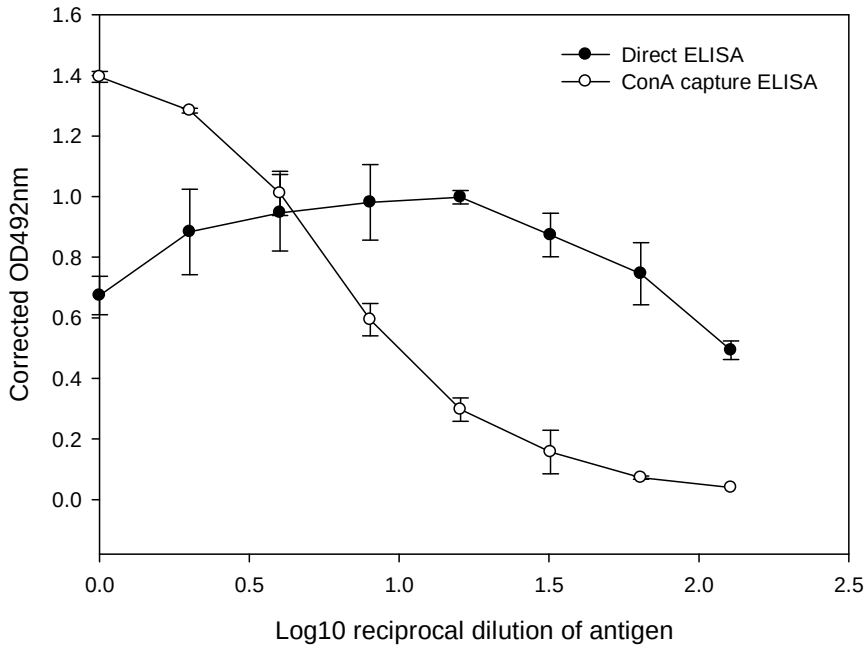


Figure 50 Comparison between direct ELISA and ConA capture ELISA demonstrated using GA2G.

The optical density of direct ELISA started off at about 0.7 and in increases slowly until an antigen dilution of 1/16 and decreasing thereafter probably suggesting the end of a plateau where the plate is saturated with antigen. The reason for the lower OD at the starting dilutions is unclear. The binding of antibody to ConA captured antigen was increased above the maximum OD that direct ELISA achieved suggesting a higher density of bound antigen at low antigen dilution.

The results showed GA2G can be captured using ConA and the antigen capture format is superior to direct ELISA in selecting for the protein of interest and boosting the signal at high antigen concentration.

6.2.3 **Coating buffer (BCB vs PBS)**

Different authors cited in section 6.2 have described the use of two different buffers for the coating of ConA namely bicarbonate coating buffer, pH9.6 (BCB) (James E. Robinson *et al.*, 1990; M. J. Robinson, 2007) and phosphate buffered saline, pH7.4. (PBS) (Clements *et al.*, 1995). This section describes the comparison between the effects of these two dissimilar coating buffers on ConA with GA2G and GA2F antigens.

GA2G, GA2F and Bgal antigens were prepared as described in section 6.2.5 and Bgal antigen was adjusted to the neat antigen concentration of GA2G and GA2F respectively.

ConA stock was diluted separately to the working concentration in either BCB or PBS. Half of the Maxisorp plate was coated with ConA diluted in BCB and the other half with ConA diluted in PBS and incubated overnight at 4°C washed as described in section 3.11.7. GA2G was diluted twofold in PBSTx and captured onto the ConA plate coated in BCB and PBS respectively in duplicate. In parallel, Bgal was coated at the equivalent concentration to GA2G. ConA capture ELISA was carried out as described in section 3.11.7 with Mab133 and GAMPx as primary antibody and secondary antibody respectively. The optical densities obtained for antibody binding to GA2G antigen were corrected by subtraction of binding at equivalent dilutions to Bgal antigen and the results are shown in Figure 51.

This experiment above was repeated with GA2F as antigen except that Mab1A12 was used as the primary antibody and the results are shown in Figure 52.

The results in Figure 51 showed that the optical densities obtained from ConA coated in PBS is higher than the optical densities obtained from ConA coated in BCB at any GA2G dilutions. This shows that PBS is a better coating buffer compared to BCB for ConA.

The results in Figure 52 supported the results obtained in Figure 51. The optical densities of ConA coated in PBS is higher than the optical densities obtained from ConA coated at BCB at any GA2F dilutions supporting that PBS is a better coating buffer compared to BCB. The results also shows that the recombinant fusion glycoprotein expressed in MVA can also be captured by ConA.

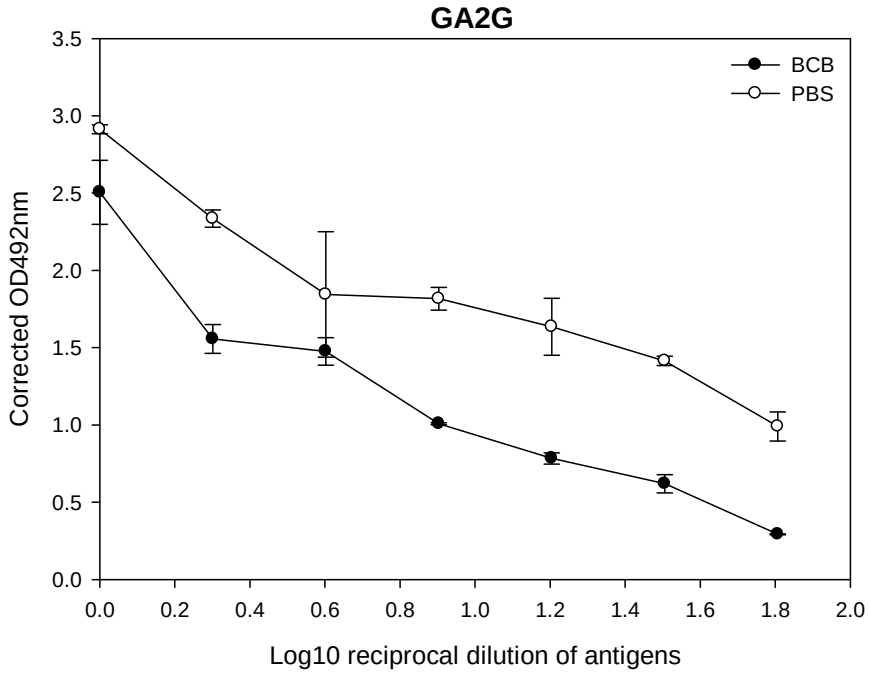


Figure 51 Comparison of coating buffer for ConA with GA2G as antigen

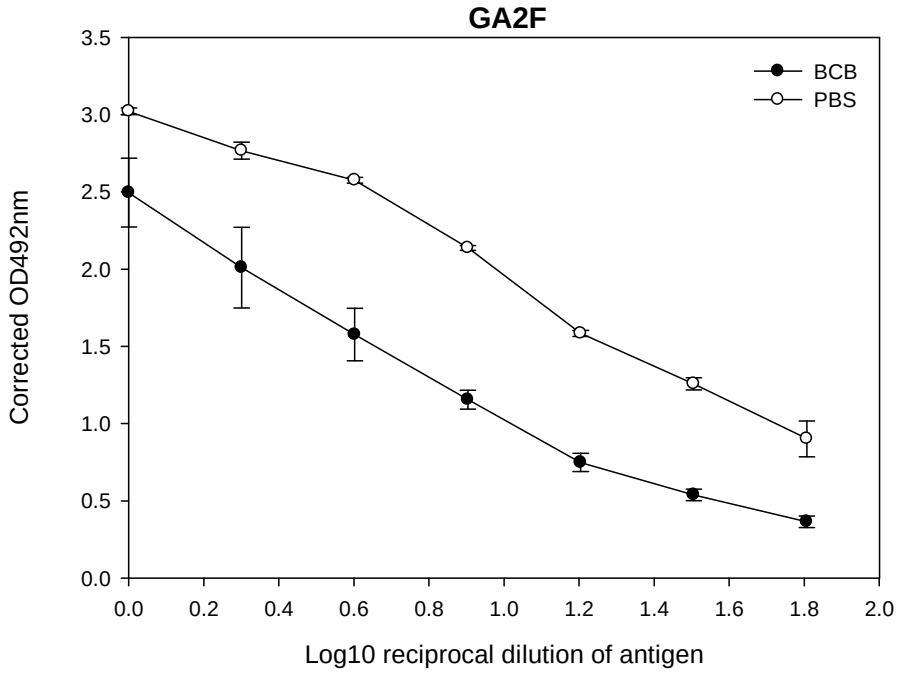


Figure 52 Comparison of coating buffer for ConA with GA2F as antigen.

ConA coating efficiency was higher in PBS than in bicarbonate coating buffer with the boost of at least 0.5 units in corrected optical density at any dilution compared as shown in Figure 52. Thus, PBS was chosen for the coating of ConA on Maxisorp plate.

6.2.4 **Serum dilutions**

Serum collected from infant volunteers is of small volume and insufficient is usually available for testing at low dilution. This section explores whether the ConA capture ELISA is of sufficient sensitivity to measure maternal antibodies to the recombinant G glycoprotein in achievable dilutions of infant sera. Two infants' sera (3810 and 1510) were chosen based on two criteria. Firstly, the infants were hospitalised due to severe HRSV disease but no NPS or NS was collected rendering them unsuitable for enrolment into the main study (see Chapter 9) and secondly, the volume of sera collected was 100 µl or more.

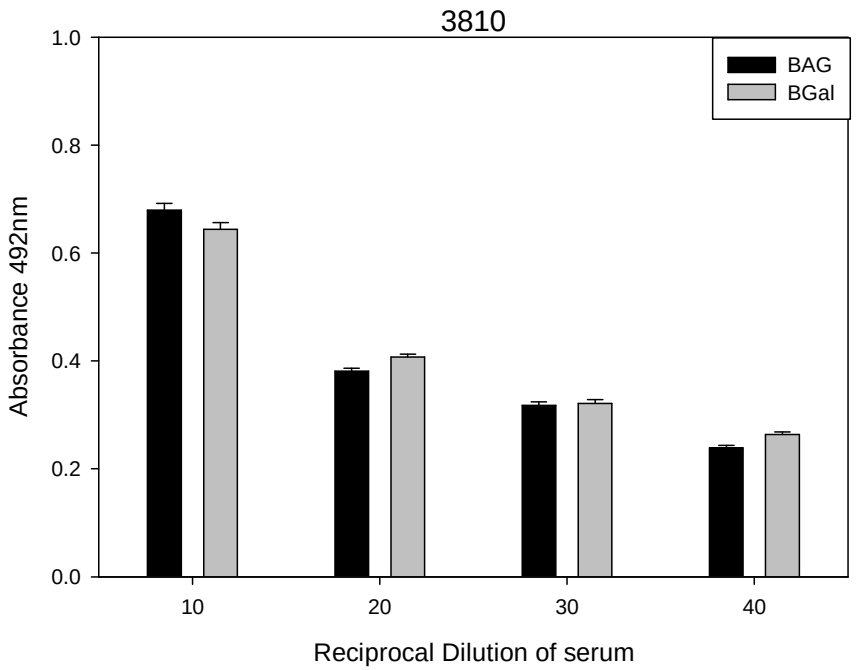
Both sera were obtained from infants with mean age of 140.5 days (both born in August 2009 and admitted in December 2009) but since NPSs or NS failed to be obtained, the infecting HRSV genotype could not be determined.

BAG and Bgal antigens were captured on a ConA plate as described in section 3.11.7. Individual sera were diluted 1/10, 1/20, 1/30 and 1/40 and assayed in duplicate on the captured antigens described above. Results are displayed as two individual bar charts in Figure 53.

Serum 3810 yielded extremely high background where the level of optical density generated from BAG and Bgal control are similar yielding a corrected optical density of approximately zero.

With reference to serum 1510, the ideal dilution used would be 1/10 but this dilution is not generally feasible due to the limited volume of serum collected from most infants. Thus, a compromised serum dilution of 1:40 was fixed for all assays. This dilution was used by Murphy et al.,(1986) for a comparable purpose.

A



B

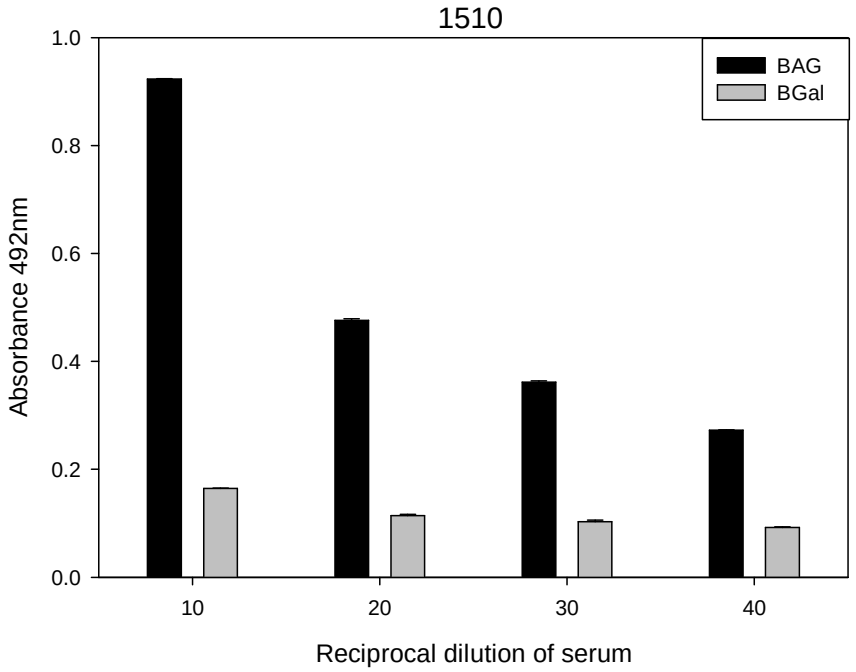


Figure 53 Reactivity of maternal antibody to the recombinant BAG and the virus Bgal virus control.

6.2.5 **Antigen preparation**

To provide a sensitive assay for the measurement of the total maternal antibody titer to the respective glycoproteins. Antigens were prepared giving a corrected optical density of 3.0 or above with either Mab133 or Mab1A12 for the G and F glycoproteins respectively.

Enriched recombinant viruses (Table 16) were propagated to passage 5 in four flasks of QT35 cells grown in T225 as described section 3.19.3 and the virus was resuspended in 3ml/flask of MM2, titered as described in section 3.19.4 and the results were shown in .

Table 16 Recombinant virus stock used to prepare recombinant proteins in HeLa cells.

Recombinant virus	Titer (pfu/ml)
vvGA2G, P5	$2.00x10^8$
vvGA2F, P5	$1.50x10^8$
vvBAG, P5	$1.25\ x10^8$
vvBgal, P5	$1.40x10^8$

The GA2G, BAG and Bgal were prepared separately in four T225 of confluent HeLa cells as described in section 3.11.5 at the MOI of 3 and the concentration determined as described in section 3.13.2.

GA2G and BAG antigens were titrated in duplicate by ConA capture ELISA on ConA plates coated in PBS with Mab133 and GAMPx as the primary and secondary antibody. The concentration of Bgal was adjusted to the neat concentration of GA2G and BAG and titrated in parallel. The optical densities obtained from the binding of antibody to GA2G and BAG was corrected by subtracting with the binding to Bgal at the equivalent concentrations and the results are shown in Figure 54.

Both antigens produced corrected optical densties of >3.0 at the lowest dilutions which are sufficiently sensitive to detect maternal antibodies in infant sera.

GA2F and Bgal antigens were prepared separately in four T225 of confluent HeLa cells as described in section 3.11.5 at the MOI of 3 and the cell debries were clarified by centrifugation at 10000g for 10 minutes at 4°C and the supernatant was recovered by aspiration. The protein concentrations were determined as described in section 3.13.2 and Bgal concentration was adjusted to the concentration of GA2F and titrated in duplicate by ConA capture ELISA on plates coated with ConA in PBS and carried out as described in section 3.11.7 with Mab1A12 as the primary antibody. The optical densities obtained from the binding of antibody to GA2F were corrected by subtracting with the binding to Bgal at the equivalent concentrations and the results are shown in Figure 55.

GA2F produced optical density of >3.0 at the lowest dilutions which is equivalent to the optical densities achieved with Mab1A12 with HRSV strain A2 infected HeLa cell lysate (section 8.2).

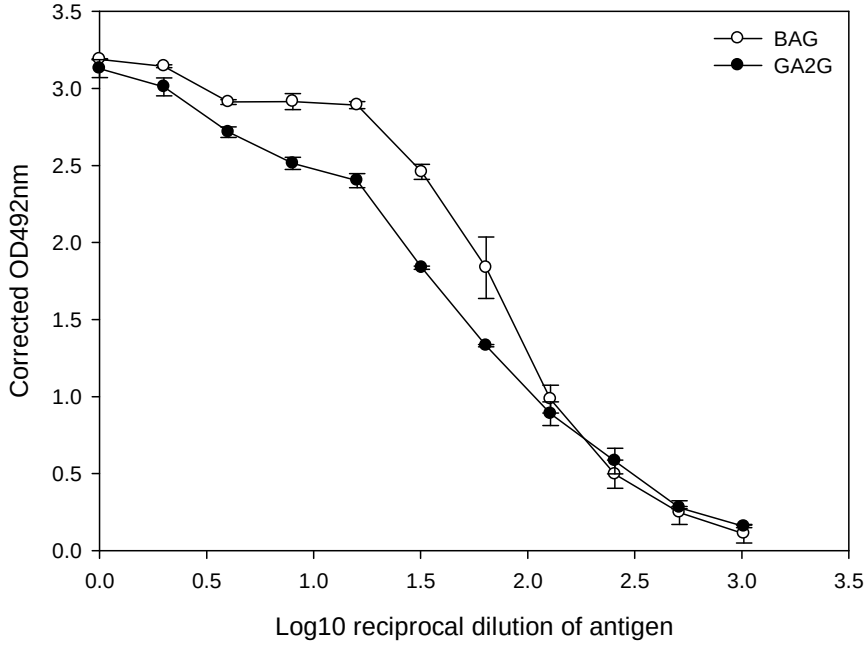


Figure 54 Antigen titration for the recombinant G glycoproteins.

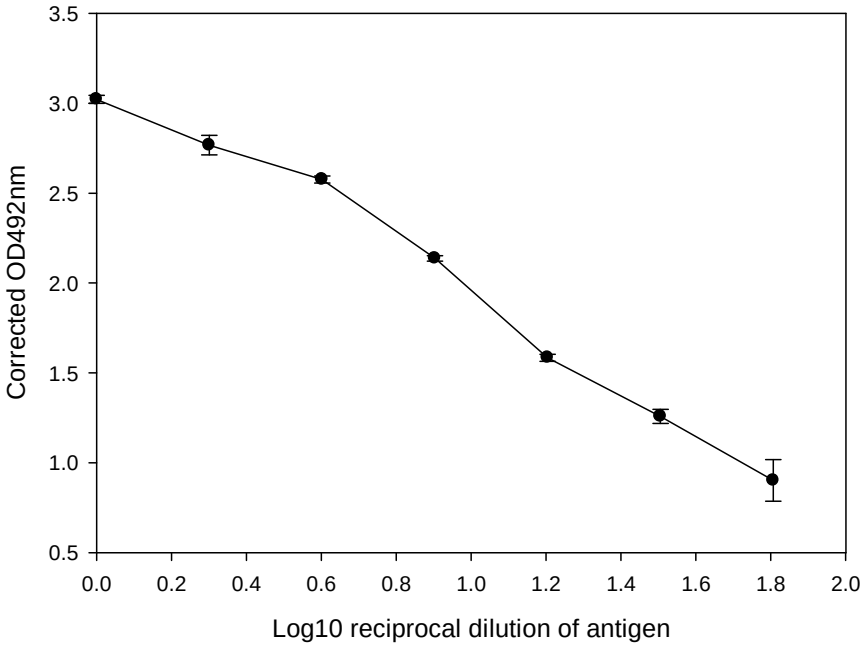


Figure 55 Antigen titration for recombinant fusion glycoprotein

Chapter 7 Development of a tool for the measurement of cross-reactive antibody

7.1 Introduction

This chapter explores the possibility of designing a tool for the measurement of antibodies to the conserved region of the G glycoprotein (Aim III) so that the antibody directed to the conserved region and hypervariable regions of the G glycoproteins can be measured separately. The subtraction of antibodies to the conserved region from total antibodies to the G glycoprotein would provide indirect measurement of antibodies directed against the hypervariable regions.

Two monoclonal antibodies were available to aid in this task. Mab133 and Mab21 were developed by Dr Mark Robinson and shown to be reactive with the G glycoprotein of both sub-groups A and B and all genotypes of both available for testing (Robinson 2007). It was reasoned that these MAbs might be used to identify the conserved epitopes on the G glycoprotein, which might then be reproduced in the form of a peptide or recombinant protein to produce suitable antigens for the detection of antibodies of similar specificity in human sera.

7.2 Peptide binding ELISA

The peptide binding ELISA experiments were carried out with Christopher Chambers, a final year undergraduate student in Microbiology at Newcastle University who submitted the results for assessment as part of his degree course.

The first approach was to test if Mabs133 and 21 recognised the highly conserved sequence in the G glycoprotein as this is the only extensive region which is fully conserved between subgroups A and B HRSV. For screening purposes, a peptide corresponding to the highly conserved amino acid sequence 164-177 was synthesized as described in section 3.21. Peptide 172-184, a mapped epitope for Mab1C2, was used as a positive control.

Peptide 164-177 was diluted to 67, 17, 4 and 1µg/ml respectively in BCB and coated onto a Maxisorp plate overnight at 4°C. Peptide 172-184 was coated onto a separate Maxisorp plate in parallel. Monoclonal antibodies 1C2, 21 and 133 were titrated four-fold in PTF and assayed on both Maxisorp plates and ELISA was carried out as described in section 3.11.6 with GAMPx as the secondary antibody. The optical densities generated due to the binding of antibodies to the respective peptides were plotted against antibody dilutions and results are shown in Figure 56.

1C2 binds to 172-187 coated at 67, 17 and 4µg/ml respectively yielding optical densities >3.0 from the lowest dilution of 1/16. Binding of 1C2 to 172-187 at 1µg/ml was lower than at the higher peptide concentrations suggesting that antibody binding is dependent on dose for concentrations of peptide below 4µg/ml.1C2 does not bind to peptide 164-177 at any peptide concentration or antibody dilution. However a slightly higher optical density was observed at the lowest antibody dilution probably due to non-specific binding of antibody to the Maxisorp plate.

Binding of 133 and 21 to 164-177 and 172-187 yielded low optical densities comparable to the binding of 1C2 to 172-187. The optical densities due to the binding of antibodies to 164-177 coated at 67µg/ml were corrected by subtracting with the binding to 172-187 coated at the same concentration. Positive values indicate binding to 164-177 while negative values indicate binding to 172-187 and the results are shown in Figure 57. The corrected optical densities for 1C2, 133 and 21 ranged between -0.093 to -3.170, 0.010 to 0.176 and -0.016 to 0.177 respectively showing that 133 and 21 did not bind to 164-177.

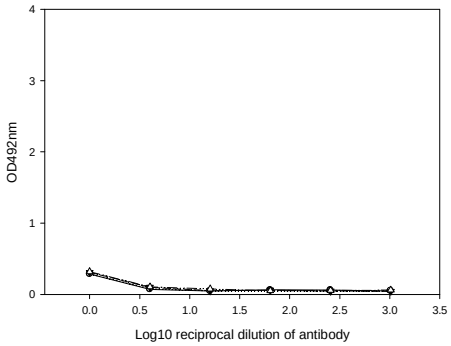
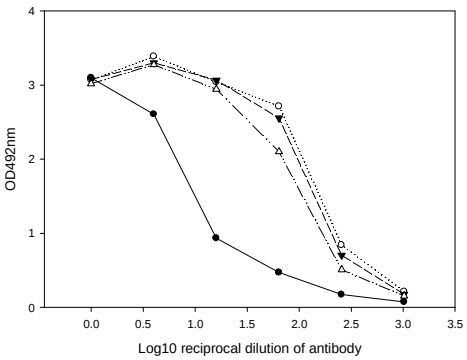
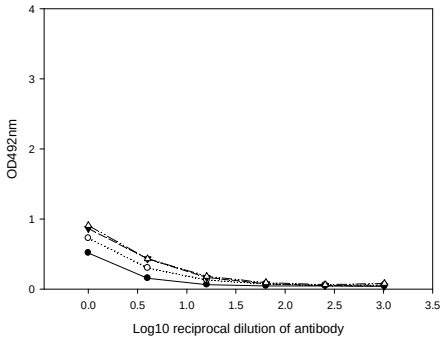
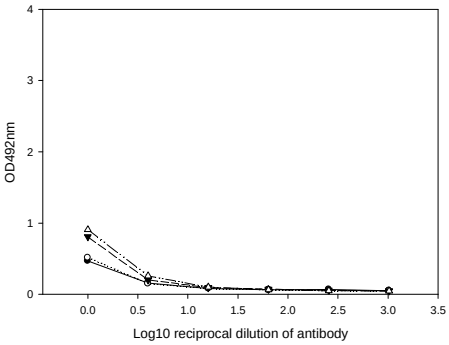
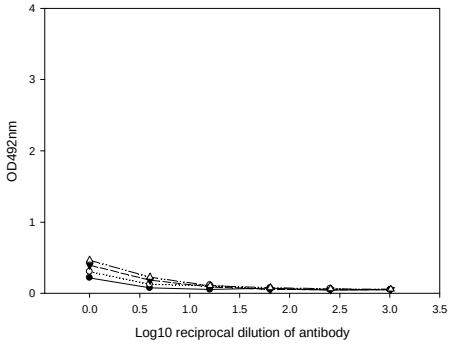
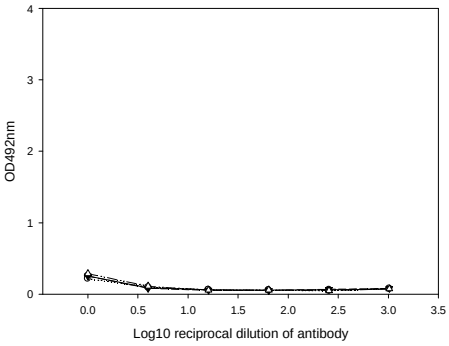
Monoclonals	Peptides	
	164-177	172-187
1C2		
133		
21		

Figure 56 ELISA of 1C2, 133 and 21 on peptide 172-187 and 164-177

●: 1μg/ml; ○: 4μg/ml; ▼: 17μg/ml;Δ: 67μg/ml

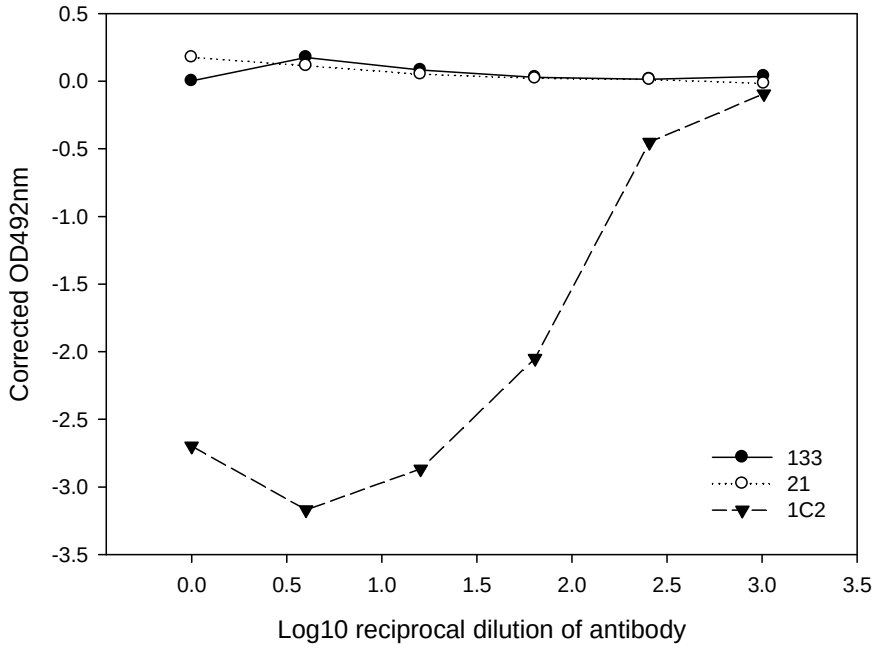


Figure 57 Corrected peptide binding ELISA

7.3 **N-glycosylation inhibition**

The previous results indicate that Mab133 and Mab21 failed to recognize a peptide corresponding to the highly conserved region of the G glycoprotein. However, the possibility remains that the antibody epitopes are in the conserved region but are dependent upon glycosylation, not present in synthetic peptides. The next step therefore was to determine whether binding of these two monoclonal antibodies is independent of glycosylation of the protein.

For this an unglycosylated form of the G glycoprotein is required which can be obtained either by the expression of the G gene in a prokaryotic expression system, by growing HRSV or recombinant virus expressing G glycoprotein in the presence of glycosylation inhibitors or by deglycosylation of mature G glycoprotein by enzyme digestion such as with endoglycosidases and PNGase F. The use of glycosylation inhibitors was adopted as being more straightforward.

The HRSV prototype strain 8/60 was selected for this purpose (and will be refered to herein as 8/60). HRSV was inoculated onto a confluent HeLa monolayer grown in a T25 tissue culture flask at the MOI of 5 and treated as described in section 3.18.3 in the presence of 1 µg/ml of Tunicamycin (MM-Tu) and harvested 48 hours post inoculation. A mock infection and untreated 8/60 cultures were conducted in parallel.

Cells were pelleted and resuspended in 100µl of 1X reducing sample buffer. All samples were heated to 95 °C in a thermal cycler and 20 µl was resolved by SDS-PAGE (section 3.12.3) and transferred to a PVDF membrane (section 3.12.4) and stained with either Mab133 or Mab21 at 1/100 and GAMPx (1/1000) as primary and secondary antibody respectively and western blotting was carried out as described in section 3.12.5 The results are shown in Figure 58. Mab1C2 was not included as it is subgroup A and does not recognise the 8/60 G glycoprotein.

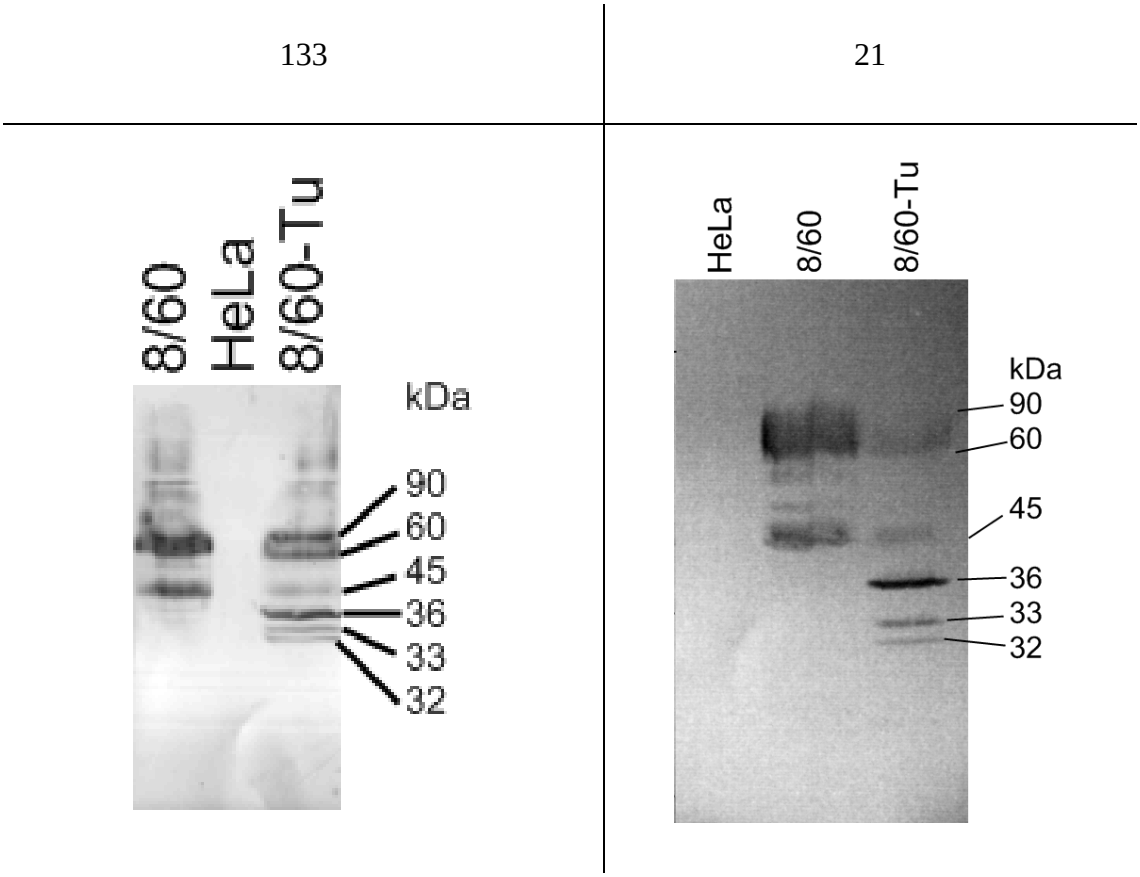


Figure 58 Tunicamycin treatment of HRSV strain 8/60.

Patterns of staining were similar for both 133 and 21. The Mabs did not recognize HeLa cell control. Untreated 8/60 shows two dominant smears of 60-90kDa and 45kDa. For Mab133 in 8/60-Tu, the 60-90kDa smear was separated into two distinct bands of approximately 80-90kDa and 60kDa. For both Mabs the 45kDa band which is present in untreated 8/60 was also present but at a much reduced intensity. Three extra bands were observed in 8/60-Tu which were not present in untreated HRSV with the molecular weight of approximately 36, 33 and 32 kDa respectively with the 36kDa band being the most intensely stained. The 36 kDa band corresponds to the electrophoretic mobility of

the unglycosylated full-sized G gene product in SDS PAGE. The fainter, smaller bands may represent truncated forms of the polypeptide (Gail W. Wertz *et al.*, 1985; Roberts *et al.*, 1994). These results suggest that tunicamycin is capable of inhibiting N-glycosylation of the G glycoprotein and reducing the efficiency in O-glycosylation and that, as Mab133 and Mab21 recognises the 36kDa precursor, they bind to the peptide backbone of the G glycoprotein rather than the carbohydrates.

7.4 **Epitope mapping**

Their apparent ability to recognize deglycosylated G glycoprotein in western blots under reducing condition suggests that these antibodies recognize linear epitopes. As they are cross-reactive between virus sub-groups, these linear epitopes must be at least partially conserved. The inability of either Mab to bind to a peptide corresponding to the conserved central region of G suggests that there is an alternative region of the polypeptide sufficiently conserved to act as a cross-reactive antibody epitope. Discovery of these regions will require epitope mapping of the Mabs. Two approaches were adopted to this end. Firstly the two cross reactive Mabs were tested together with other Mabs to G in additive binding assays in order to assess how many epitopes are involved. Secondly, the Mabs were reacted with truncated forms of the G glycoprotein, transiently expressed under the control of T7 RNA polymerase promoter to locate the epitopes on the polypeptide chain.

7.4.1 **Additive ELISA**

In the additive ELISA for epitope mapping, Mabs are tested in pairs to determine whether they compete for the same epitope or bind additively to two separate epitopes. The concentration of target antigen is adjusted so that the optical density produced by antibody binding in the presence of excess antibody is approximately 1.0. In antibody excess, doubling the concentration of either Mab or mixing the two Mabs which bind to the same epitope does not increase the amount of antibody bound. Mixing two Mabs which bind to different epitopes increases the amount of antibody bound by a maximum of 2-fold. MAbs 21 and 133 were tested together with MAb 1C2, known to bind to a defined sub-group specific epitope, and MAbs 4G4 and 3F43 which bind an undefined epitope present on only some sub-group A strains but which have previously been shown to bind additively (Routledge *et al.*, 1986) and are thus regarded as positive controls.

Additive binding ELISA was carried out in triplicate with anti-G monoclonal antibodies 133, 21, 1C2, 4G4 and 3F43 as described in section 3.11.8 and the results are shown in Figure 59. The effective dilutions used for 133, 3F43, 4G4, 21 and 1C2 was 1/256, 1/2048, 1/8, 1/128 and 1/256 respectively. The concentration of HRSV strain A2 antigen prepared as described in section 3.11.4 were coated in BCB at 1/1000 dilution, a dilution previously determined to give an OD of approximately 1.0 with the mentioned dilution of Mabs. Mixtures comprise of equal volumes a double concentration of each Mab.

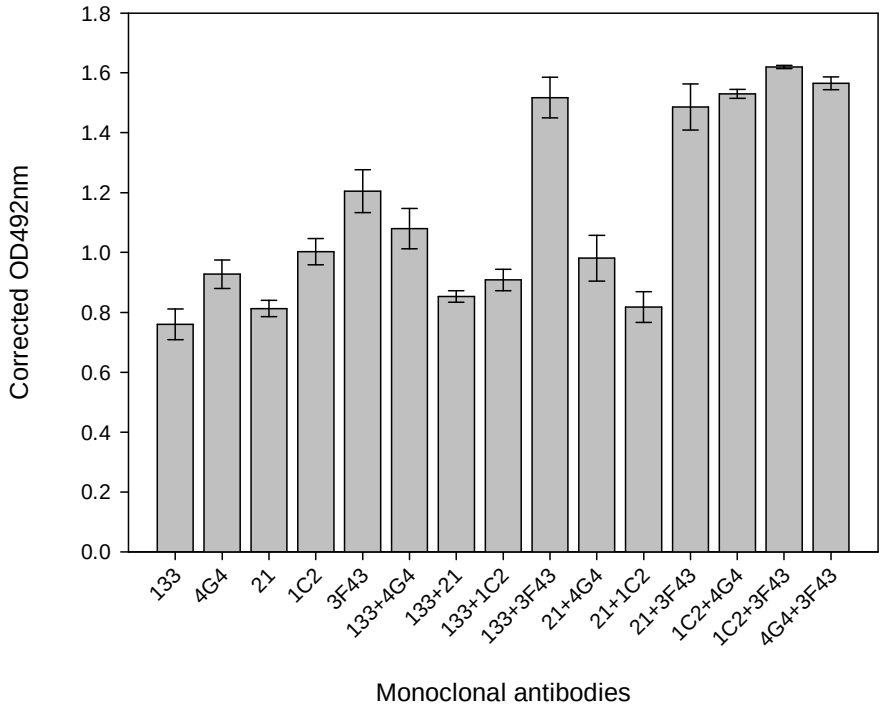


Figure 59 Additive binding ELISA.

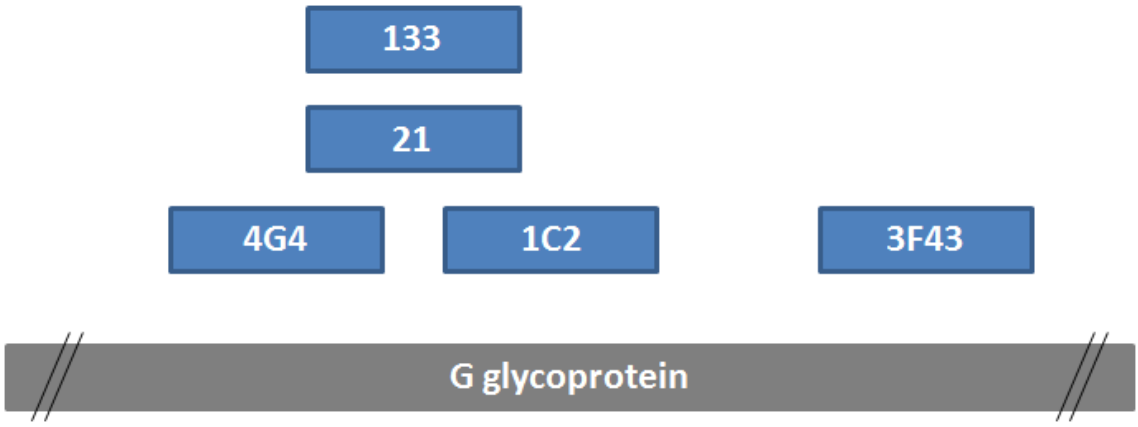


Figure 60 Epitope relationship between monoclonal antibodies

The relationship of the monoclonal antibodies tested is summarized in Figure 60. Mab133 and Mab 21 were competing for the same or overlapping epitopes. Part of this epitope is shared by Mab1C2 and Mab4G4 independently. Mab1C2 and Mab4G4 were found to bind to two distinct epitopes judged by the additive binding effect (p=0.002) whereas Mab3F43 binds to a distinct epitope from Mab133 (p=0.014), Mab21 (p=0.011), Mab1C2 (p=0.011) and Mab4G4 (p=0.013) (statistical analysis was carried out using paired t test as described in section 3.23).

7.5 **Truncation of G**

To facilitate the further definition of epitopes on the G glycoprotein, the full gene and truncated forms of it were cloned into plasmid pTM3 downstream of a T7 promoter and expressed transiently in HeLa cells under the control of T7 RNA polymerase supplied by superinfection with a recombinant MVA expressing that enzyme.

All full length and truncated G gene products were amplified from plasmid clone 25173G2/pSC11_1. A miniprep plasmid of 25173G2/pSC11_1 was diluted 1/1000 in DEPC-H_20 and PCR was carried out using the primer pairs in Table 17 as described in section 3.15.4 using programme PCR55 and resolved in an agarose gel as described in section 3.15.9. The truncated forms of the polypeptide generated are presented schematically in Figure 61. The results are shown in Figure 62 and Figure 63.

Table 17 Truncation primer sets

Construct	Forward primer	Reverse Primer	Length (bp)
A	G-N151F	G284R	423
B	G-N155F	G284R	411
C	G-N158F	G284R	402
D	G-NcoIF	G177R	558
E	G-NcoIF	G172R	543
F	G-NcoIF	G284R	876
G	G-NcoIF	G190R	597
H	G-NcoIF	G163R	516

The number in the forward primer indicated the start of the amino acid corresponding to strain 25173 of genotype GA7 (A. McGill *et al.*, 2004b) and the complete G gene sequence is shown in section 12.6. The number in the forward primer and reverse primer indicated the starting and last amino acid position in the G gene.

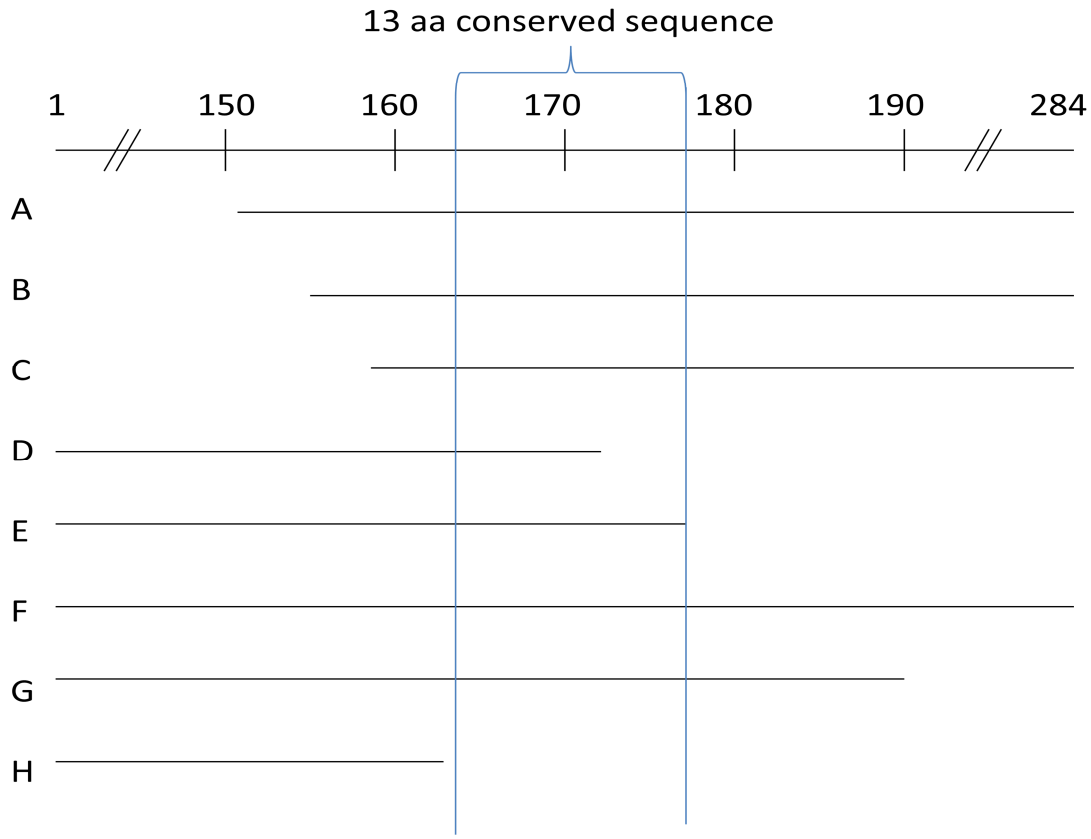


Figure 61 Schmatic diagram showing the representation of truncated products compared to the full length G glycoprotein of GA7G (construct F).

Note: Truncate G and H were purposely not displayed in increasing length with the truncate D, E and F for ease of comparison with Figure 62 and Figure 63.

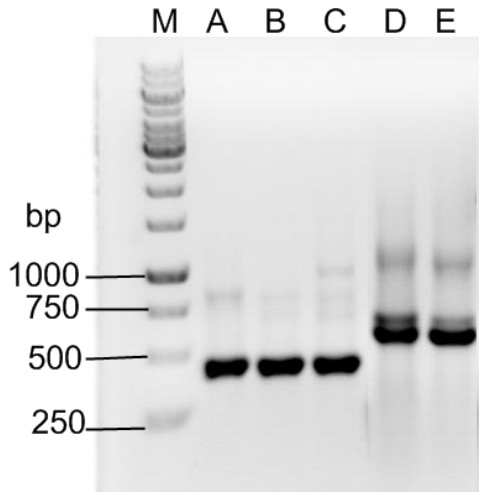


Figure 62 Truncation PCR I

Lane alphabets corresponds to the primer pair used in Table 17

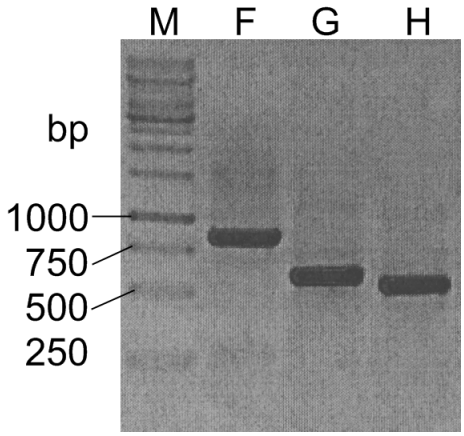


Figure 63 Truncation PCR II

Lane alphabets corresponds to the primer pair used in Table 17

Constructs A, B and C yielded a dominant band in between 250-500 bp corresponding to the expected size of 423bp, 411bp and 402bp respectively with non specific bands observed in between 750-1000bp. Constructs D and E yielded bands corresponding to the expected size of 558bp and 543bp respectively with non-specific bands observed above the band of interest.

Figure 63 shows the amplification of the full length G gene, construct F, with the expected size of 876bp. Constructs G and H yielded bands corresponding to the expected size of 597bp and 516bp respectively. All yielded single bands without any visible non-specific bands.

7.5.1 **Preparation of pTM3 for cloning**

A maxiprep plasmid of pTM3 was prepared as described in section 3.17.11 and digested with NcoI and BamHI separately as described in section 3.17.3.

7.5.2 **Cloning into pTM3**

All bands of interest from section 7.5 were excised and gel purified as described in section 3.15.10, digested with NcoI and BamHI as described in section 3.17.3 and ligated into pTM3 prepared in section 7.5.1 as described in section 3.17.5 followed by transformation into chemically competent TG1 (section 3.17.9).

7.5.3 **Insert screening**

Insert screening by colony PCR requires the use of colony PCR primer sets which bind to the vector sequence. As for pSC11, there is no published sequence for pTM3 or pTM1 in the public domain. Thus, a sequencing primer pair was designed complementary to the multi cloning site (MCS) (B. Moss *et al.*, 1990) and named pTMvec_F and pTMvec_R (Table 8). pTM3 was sequenced using pTMvec_F and pTMvec_R as described in section 3.15.13 and the colony PCR primers binding sequences were manually designed based on the sequences obtained and were named pTM-coF and pTM-coR. pTM3 was sequenced with pTM-coF and pTM-coR and the sequences were assembled together with sequences obtained with primers pTMvec_F and pTMvec_R and are shown in Figure 64.

AAGCGTATTCAACAAGGGGCTGAAGGATGCCCAGAAGGTACCCCATTGTAT
GGGATCTGATCTGGGGCC**TCGGTGCACATGCTTTACAT**GTGTTTAGTCGAG
GTTAAAAAAACGTCTAGGCCCCCCGAACCACGGGGACGTGGTTTTCCTTTG
AAAAACACGATAATACC*ATG*GGAATTCCCCGGGGAGCTCACTAGTGGATCC
CTGCAGCTCGAGAGGCCTAATTAATTAAGTCGACGATCCGGCTGCTAACAA
AGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATA**ACTAGC**
ATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGG
AGGAACTATATCCGGGTCCGGAGTTAACTCGACATCTATATACTATATAGTA
ATAC

Figure 64 Partial pTM3 sequence.

Bold: pTM-coF and pTM-coR binding site respectively; underlined: multicloning site (B. Moss *et al.*, 1990); italiced start codon.

In order to identify colonies with and without inserts on which to validate the colony PCR primer set, plasmid minipreps of the resulting colonies from experiment F, G and H (alphabets according to Table 17, PCR product according to Figure 63, cloned into plasmid pTM3 and transformed into TG1 as described in section 7.5.2) were prepared as described in section 3.17.10 and digested with NcoI and BamHI separately as described in section 3.17.3 and the resolved bands are shown in Figure 65.

Colonies 1-4 from experiment F contain insert of the correct size of approximately 876bp, colony 5 did not harbour any visible plasmid on gel and colony 6 contains no insert. All of the eight colonies picked for experiment G (aa1-190) yielded positive insert with the expected size of about 597bp. All 7 clones from experiment H (aa1-163) harbours insert with the expected size of 516bp except colony 6 which contains no plasmid.

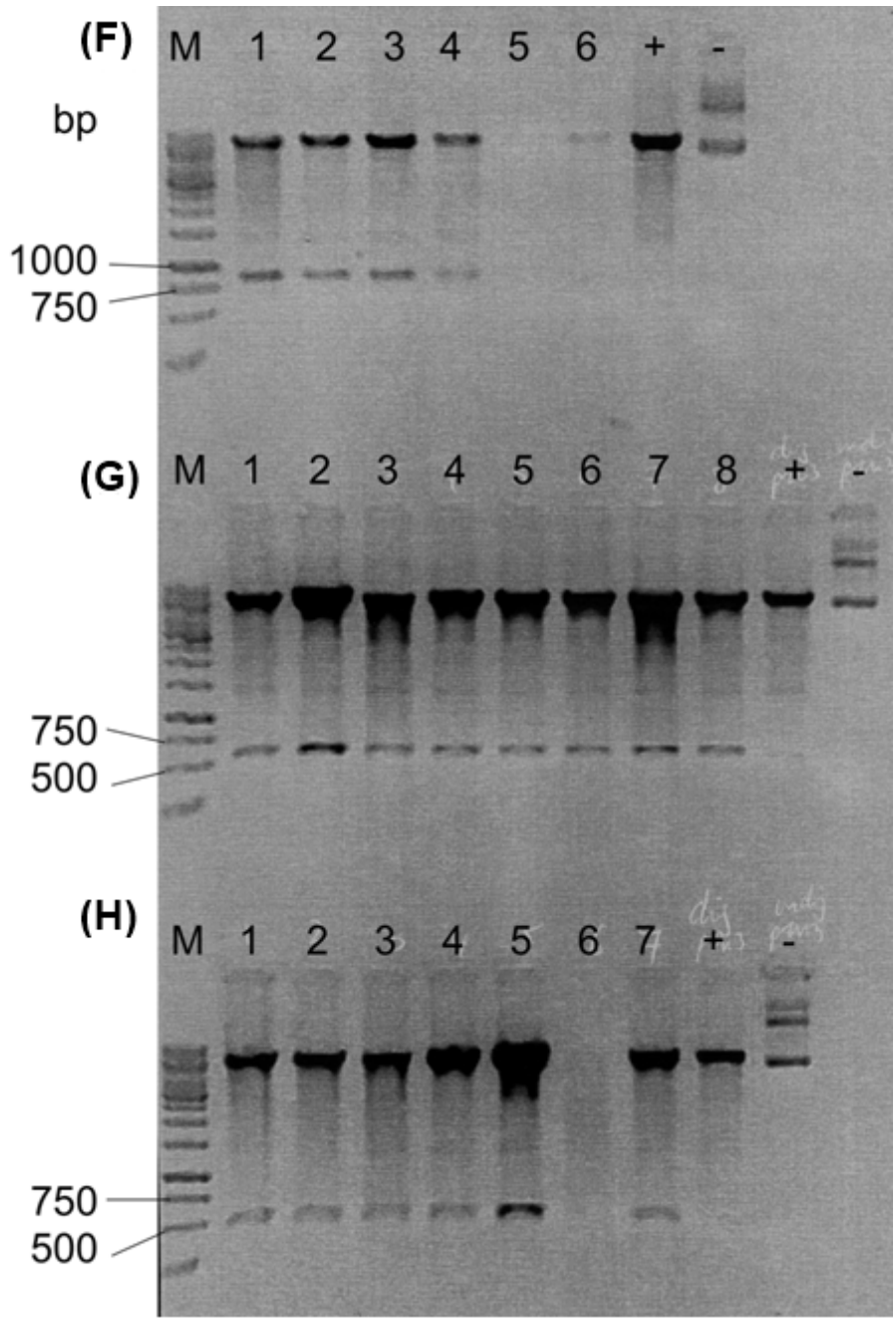


Figure 65 Colony screening by NcoI and BamHI digestion.

M: 1kb DNA ladder, Numbers representing clone/colony number; + RE digested pTM3, - undigested pTM3. Alphabets corresponding to the primer set used in Table 17

Colony 1 from experiment F, G and H were streaked on LBA plates followed by overnight incubation at 37°C to obtain single colonies. A single colony was picked for the plate and colony PCR was performed with pTM-coF and pTM-coR with programme PCR50 as described in section 3.15.6. All amplicands showed the expected profiles on agarose gel (Figure 66) with the insert PCR product (as shown in Figure 63) plus an additional 251bp corresponding to the vector sequence flanking the insert. pTM3 vector control yielded a band corresponding to the expected size of 251bp in relation to the marker. The water control was clean.

These results showed that the colony PCR primers set can be used for the screening of inserts in recombinant pTM3 clones.

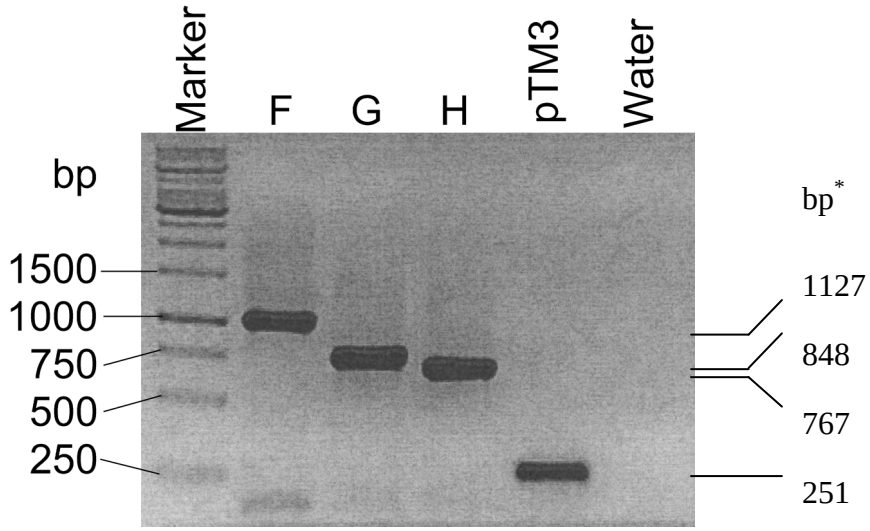


Figure 66 Validation of the primer set for colony PCR on pTM3 and its constructs.

F, G, H: colony 1 from clones derived from Figure 65; pTM3: vector control, Water : template free control. * expected size.

Subsequently, nine bacterial clones each resulting from section 7.5.2 corresponding to experiment A, B, C, D and E (Table 17) were picked and colony PCR was carried out using the validated primer set as described in section 3.15.6, resolved in agarose gel as described in section 3.15.9 and the results are shown in Figure 67. A bacterial clone containing pTM3 plasmid was picked and included in the colony PCR as a control.

All nine colonies from truncation experiments A, B and C screened for inserts by colony PCR yielded a single band of the expected size of about 674, 662 and 674bp respectively. Seven of nine clones of experiment D yielded a single band of the expected size of 809bp with clones 5 and 9 yielding a band lower than the expected size.

Seven of nine clones from experiment E showed a band corresponding to the approximate expected size of 794bp with clones 1 and 9 yielding a band smaller than the expected size. The pTM3 control showed a band corresponding to the expected size of 251bp.

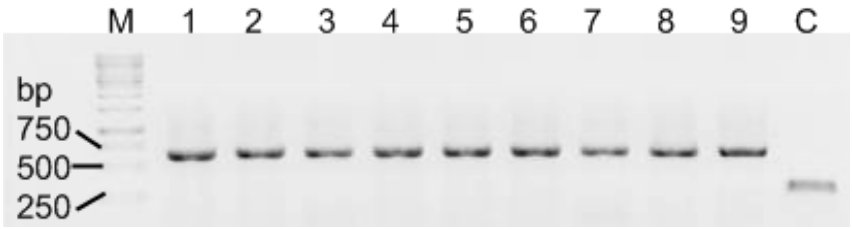
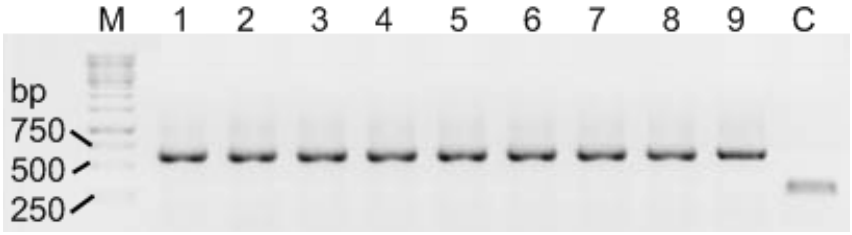
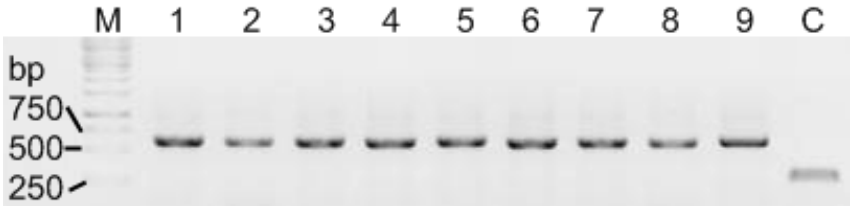
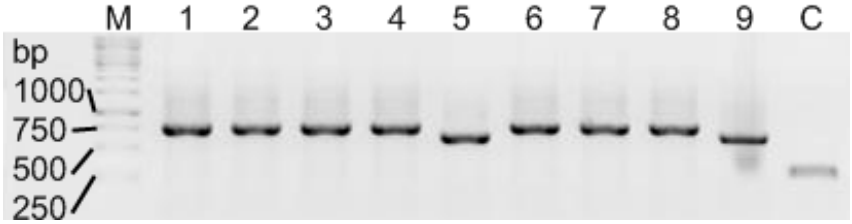
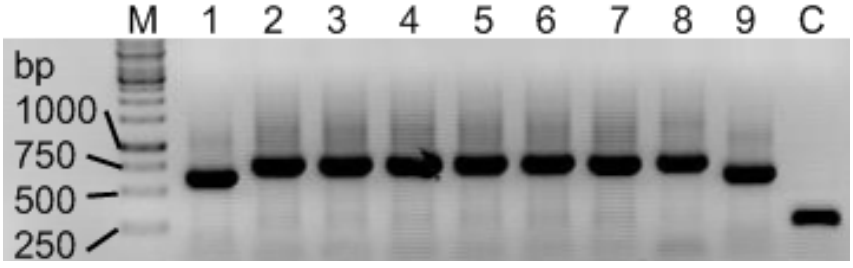
Primer pairs	Gel Image
151-284 (A)φ	
155-284 (B) φ	
158-284 (C) φ	
NcoIF-177 (D) φ	
NcoIF-172 (E) φ	

Figure 67 Insert screening for the truncated G gene in pTM3.

φAlphabets representing experiments in Table 17, M: 1kb ladder; C: pTM3 control.

7.5.4 **Plasmid preparation**

The first positive clones from each experiment from section 7.5.3 including the bacterial clone containing pTM3 plasmid were selected. Plasmid maxipreps were carried out as described in section 3.17.11 and the concentration of each plasmid was determined as described in section 3.15.12 and the results are shown in Table 18

Table 18 Concentration of plasmid DNA of pTM3 clones

Clone name	Concentration (ng/µl)
1-284	1900
1-190	4000
1-163	308
1-172	665
1-177	1000
151-284	1700
155-284	1600
158-284	1200
pTM3	2800

7.5.5 **Transient expression**

The recombinant G glycoprotein gene and its truncated products in pTM3 plasmid listed in Table 17 were transiently expressed in HeLa cells superinfected with MVAT7 as described in section 3.19.8. pTM3 transfected cells, MVAT7 infected cells and uninfected HeLa cells were included as controls. The monolayer was scraped into the medium 24 hours p.i. and slides were prepared as described in section 3.10.3.

A nearly confluent monolayer of HeLa in T75 was infected with HRSV strain 25173 (refered to herein as HRSV GA7 as described in section 3.18.3. A mock infection was carried out in parallel. When 4+ CPE was observed, the monolayer was scraped into the medium and was fixed onto slides as described in section 3.10.3. These slides were included in immunofluorescence tests as controls.

7.5.6 **Antibody optimization for Immunoflourescence**

Prior to testing anti-G Mabs on truncated G antigens it was necessary to determine the dilution of each MAb preparation by immunofluorescence staining of HRSV positive control antigen.

A confluent HeLa monolayer in a T75 flask was infected with HRSV strain A2 as described in section 3.18.3 and cultivated until 4+ CPE was observed. A mock infected HeLa monolayer was prepared in parallel. The monolayer was scraped into the medium and smears prepared on microscope slides and fixed (section 3.10.2) as described in sections 3.10.3. Anti-G monoclonal antibodies (Mabs 1C2, 21, 133, 4G4)

were titrated on the fixed antigen in PBS and immunostaining was carried out as described in section 3.10.4 and the results are shown in Table 19

Table 19 Antibody dilutions against HRSV strain A2 by IF.

	Antibody dilutions				
Mabs	1/2	1/4	1/8	1/16	1/32
21	4+	4+	4+	4+	4+
1C2	4+	4+	4+	4+	3+
133	4+	4+	4+	3+	2+
4G4	4+	4+	4+	2+	1+

Mabs 1C2, 133, 21 and 4G4 were used at 1/16, 1/8, 1/32 and 1/8 respectively for subsequent immunofluorescent detection of recombinant G glycoproteins and its truncates in section 7.5.7.

7.5.7 **Immunofluorescence staining of anti-G Mabs on truncated G antigens**

Specimen slides prepared from section 7.5.5 were stained with Mabs 133, 21, 1C2 and 4G4 and immunofluorescent procedures were carried out as described in section 3.10.4 and the results are shown in Figure 68. An example of immunoflourescent staining with Mab133 on transiently expressed G glycoproteins is shown in Figure 68.

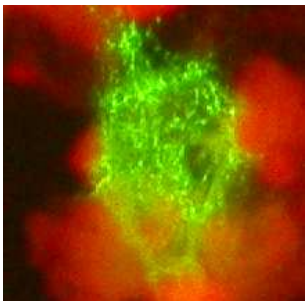
Positive	Negative
	

Figure 68 Immunofluorescent staining on transiently expressed G glycoprotein.

Truncated fragment 1-284 (full length G) transiently expressed in HeLa cell and stained with Mab133 followed by SAM-FITC counterstain and visualized under 400X magnifications.. Apple green staining represents positive IF while red background represents negative IF.

Table 20 Monoclonal antibodies profile to the truncated G protein fragments by immunofluorescence.

	Truncation from N-terminus			Truncation from C-terminus					Controls			
Mabs	151-284	155-284	158-284	1-163	1-173	1-177	1-190	1-284	pTM3	MVA-T7	GA7	HeLa
133	4+	4+	-	-	-	-	4+	4+	-	-	4+	-
21	4+	4+	1+	-	-	-	4+	4+	-	-	4+	-
1C2	4+	4+	4+	-	-	-	4+	4+	-	-	4+	-
4G4	4+	4+	1+	-	4+	4+	4+	4+	-	-	4+	-

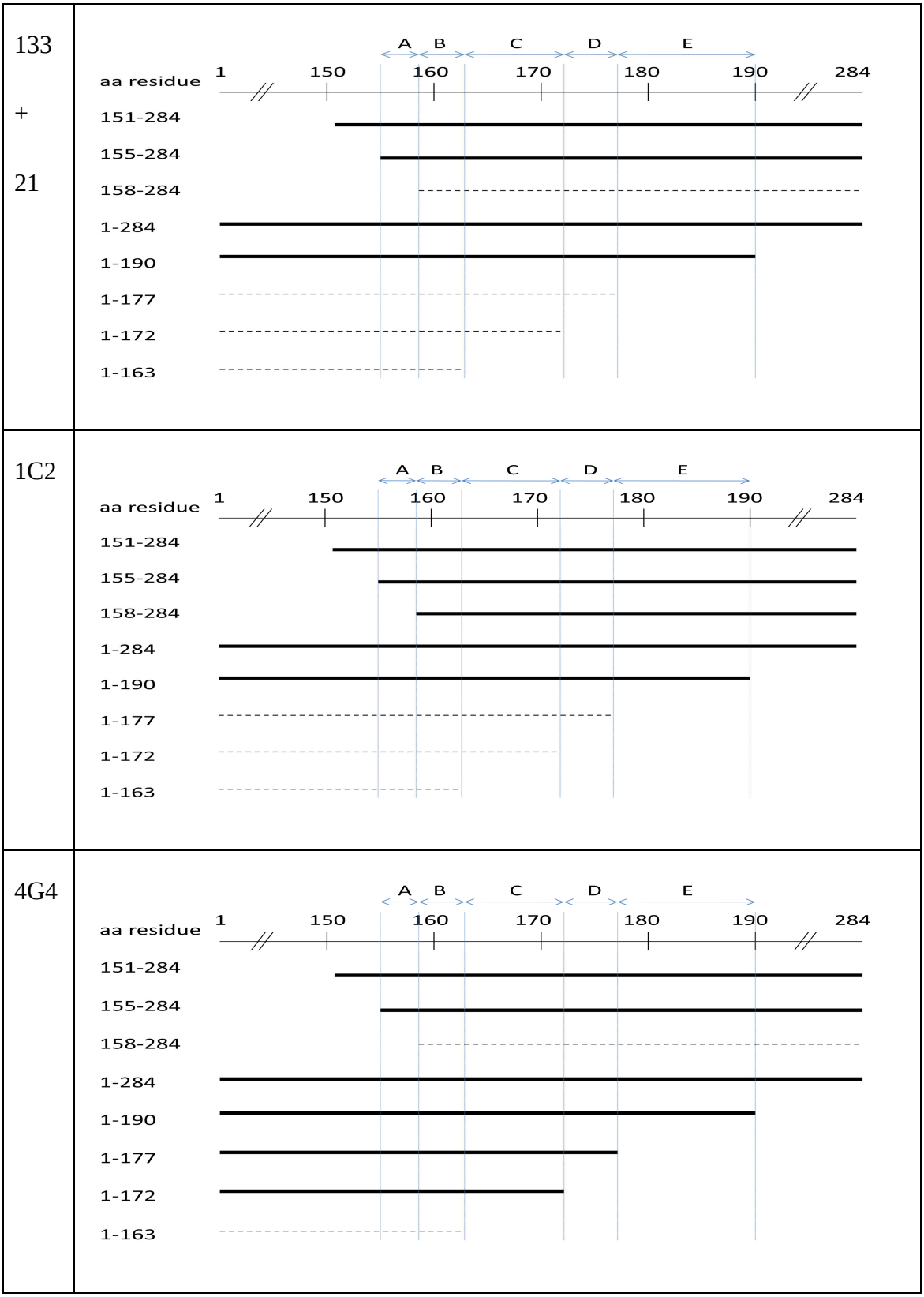


Figure 69 Schematic diagram showing the binding of the monoclonal antibodies to the G glycoprotein and its truncated fragments.

Solid lines and hatched lines represent binding and no binding respectively.

To analyse the binding of each monoclonal antibody to the G glycoprotein and its truncated products, the G glycoprotein was divided into five regions represented with the alphabets A to E as shown in Figure 69.

Both Mab133 and Mab21 displayed a similar binding profile to the truncated fragments and will be analysed together. The epitope of both Mabs were mapped within residues 155-190 spanning 45 amino acids in length while further truncation of the 3 residues from the C-terminus end within region A (Figure 69) fully ablated the binding of Mab133 and weakened the binding of Mab21 suggesting that at least one of the three amino acids is important for the binding of these two cross-reactive Mabs which may bind to different overlaping epitopes within the same region. At the N terminus end, region E is important for the binding of both Mabs. Lack of binding to fragments 1-177 and 185-284 which contains the complete 13 amino acids conserved sequence (164-177) reinforced the results of the peptide binding ELISA which suggested that residue 164-177 is not sufficient for the binding of these two cross-reactive Mabs.

The truncation experiment narrowed down the epitope of subgroup A specific monoclonal Mab1C2 to 155-190 which contains the known epitope of residues 172-184. Removal of region E completely ablated the binding of Mab1C2 to the truncated fragment.

The epitope for the strain-specific monoclonal antibody Mab4G4 was narrowed to residue 155-172 which requires region A and C at the C terminus and N terminus end respectively for efficient binding. Similar to Mab21, the deletion of region A causes Mab4G4 to bind weakly judging by the 1+ IF to fragment 158-284.

Within region A, residue 155 and 156 consist of two proline residues which are conserved in both subgroups A and B HRSV. Residue 157 is variable being serine or asparagine residue in subgroup A or a conserved lysine residue in subgroup B. Thus, residue 157 is the only variable residue in the Mab4G4 epitope it is likely to be involved in binding of that antibody conferring strain specificity. At least one of the two conserved prolines are likely to be involved in binding of the cross-reactive Mab133 and Mab21.

Region E, essential for binding of cross-reactive Mab133 and Mab21, and also of sub-group A specific Mab1C2, contains conserved residues 178, 179, 182, 185-187, 189, 190 while residues 177, 180, 181, 183, 184 and 188 vary between but are conserved

within subgroups (Table 21)). It is surmised that one or more of the conserved residues contribute to the binding of Mab21 and Mab133 whilst at least one variable residue is important in the binding of Mab1C2.

Table 21 Differences between amino acid residues within region E in the G glycoprotein.

		Amino acid residues				
Subgroup	177	180	181	183	184	188
A	Ser	Pro	Thr	Trp	Ala	Arg
B	Gly	Gln	Leu	Lys	Ser	Thr

Subgroup specific Mab4G4 and subgroup A specific Mab1C2 binds to distinct epitopes in the G glycoprotein while the cross-reactive monoclonal antibodies Mab133 and Mab21 binds to overlapping epitope shared by Mab4G4 at the C terminus end and Mab1C2 in the N-terminus end missing the 13 amino acids conserved sequence in the middle.

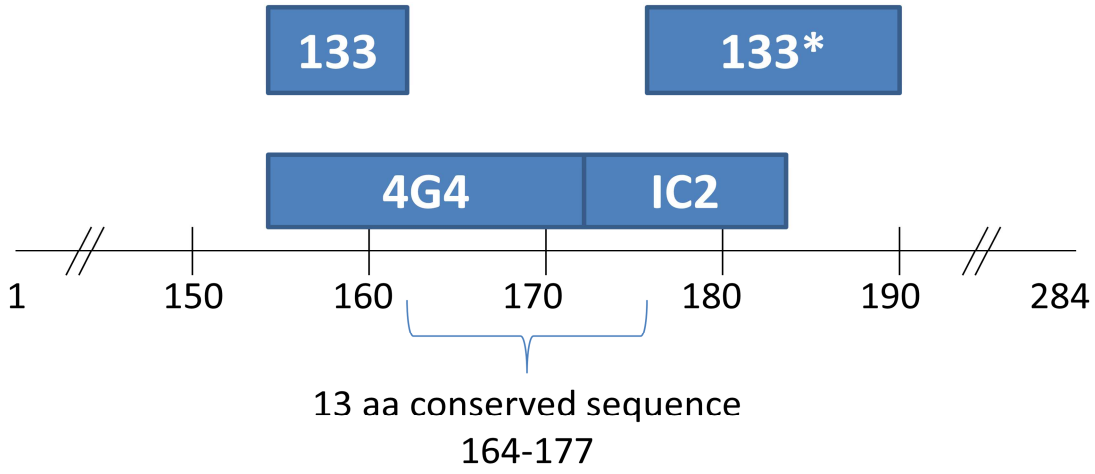


Figure 70 Epitopes for cross-reactive, subgroup specific and strain specific monoclonal antibodies in the G glycoprotein.

The overall relationship between these cross-reactive, subgroup specific and strain specific monoclonal antibodies are summaried in *including Mab21.

Thus, the design of a straight forward antigen tool for the measument of maternal antibodies directed to the cross-reactive epitopes is not feasible without measuring the subgroup specific and strain specific antibodies.

Chapter 8 Screening for Immunoglobulin A (IgA) in infants

8.1 **Introduction**

As maternal IgG is indistinguishable from an infants' own IgG, the screening of IgA in infants' sera collected from epidemic 2009/2010 was crucial. The presence of IgA antibody in the serum indicates that the infant has already mounted its own humoral response against HRSV and that there is a possibility that IgM and/or IgG against HRSV glycoprotein might have been produced to the current or previous infection. Thus, all IgA positive infants were excluded from the study.

8.2 **Mab1A12**

Development of an ELISA for anti-HRSV IgA requires prior optimization of an HRSV antigen, testing of known HRSV IgA positive sera and demonstration of the specificity of the assay for IgA immunoglobulins. Optimization of the ELISA antigen was carried out using anti-RSV Mab1A12, which was first titrated on an arbitrary dilution of antigen.

HRSV strain A2 (section 3.18.1) (refered to hereafter as HRSV antigen) was inoculated into 4 confluent T75 of HeLa cells and antigens were prepared as described in section 3.11.4. A mock infected cell control was prepared in parallel as a control (refered to hereafter as cell control). The protein concentration of each antigen was determined as described in section 3.14.1.

Half of a Maxisorp plate was coated with HRSV infected HeLa cell lysate and the other half with uninfected HeLa cell lysate at the concentration of 20 µg/ml in BCB and incubated overnight at 4 °C. Mab1A12 was serially diluted twofold on each antigen and direct ELISA was carried out as described in section 3.11.6 with GAMPx as the secondary antibody. The assay was carried out in duplicate. The optical densities from the binding of antibody to HRSV antigen was corrected by subtraction of binding to the uninfected cell control and the results are shown in Figure 71.

The corrected optical densities plateaued from dilution 1/4000. Thus, a 1/2000 dilution within the plateau region was fixed as the optimal concentration for Mab1A12 in ELISA.

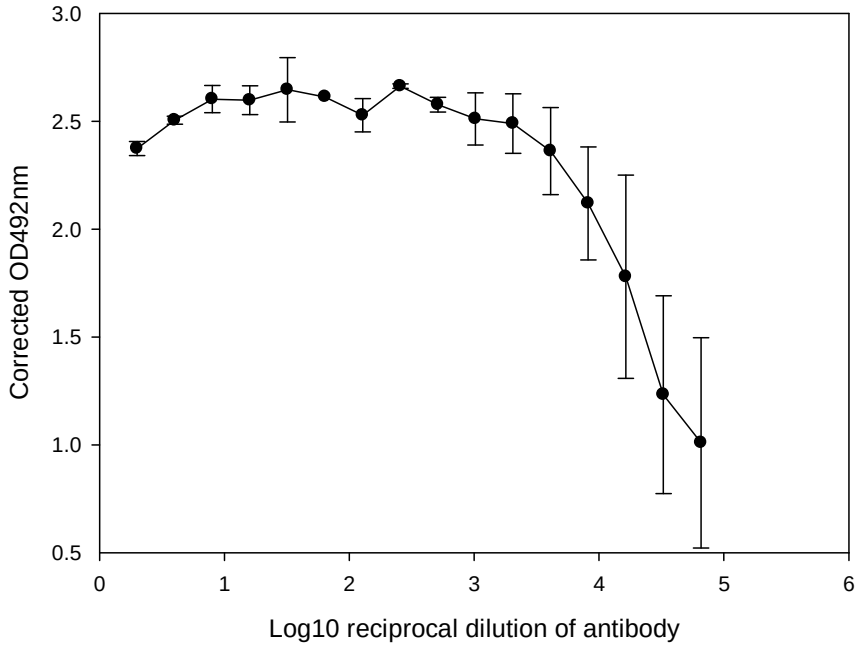


Figure 71 Optimization of Mab1A12 on a direct A2 ELISA.

8.3 A2 antigen concentration optimization

HRSV antigen and uninfected cell lysate (section 8.2) were titrated in parallel in duplicate in 50µl/well of BCB and uninfected cell lysate was titrated in parallel in a Maxisorp plate followed by overnight incubation at 4°C. Direct ELISA was carried out as described in section 3.11.6 with 1A12 and GAMPx as the primary and secondary antibody respectively. 1A12 was diluted 1/2000 in PTF as optimized in section 8.2. The optical densities resulting from the binding of antibody to HRSV antigen were corrected by subtracting the binding to the uninfected HeLa cell lysate at the equivalent concentration and the results are shown in Figure 72. The corrected optical density plateaued from 1/256 dilution and dropped thereafter. The optimal antigen coating concentration should fall within the plateau and was fixed at 1/200 corresponding to a coating concentration of 50 µg/ml total protein.

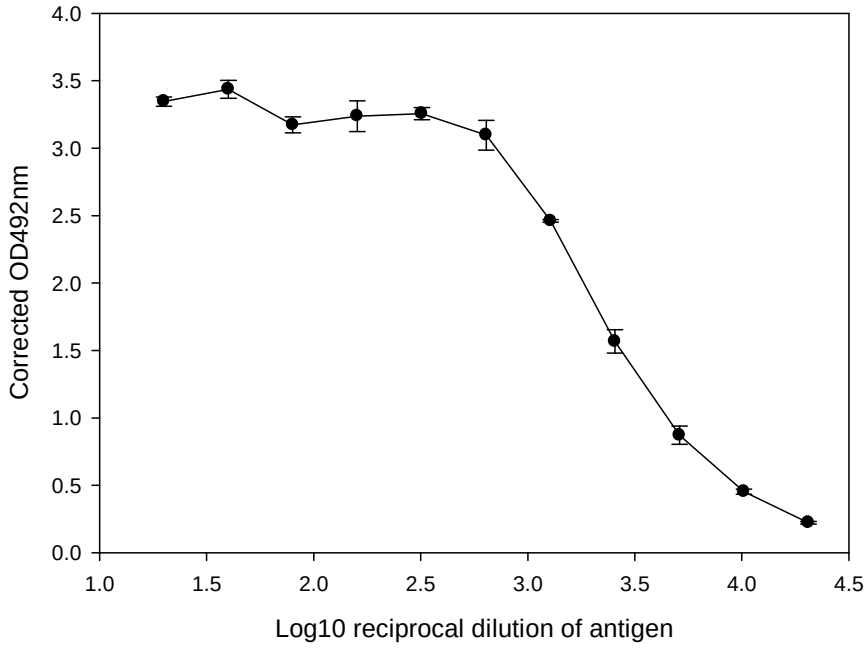


Figure 72 Optimization of A2/HeLa coating concentration.

8.4 IgA Controls

In order to screen for IgA in infants' sera, a known anti-HRSV IgA is required as control. Anti-HRSV IgA can be found in colostrum and expressed breast milk, serum and nasal secretions (Mark Fishaut *et al.*, 1981; Nandapalan *et al.*, 1987; Yamazaki *et al.*, 1994; de Alarcon *et al.*, 2001) but in this section, NPS collected for virus isolation during the 2008/2009 epidemic was chosen due to its availability.

Half of a Maxisorp plate was coated with 50 µg/ml of HRSV infected HeLa antigen prepared in section 8.2 and the other half with the equivalent concentration of uninfected HeLa cell antigen as described in. section 3.11.6.

Due to the unknown quantity of nasal secretions and the dilution factor (in Hank's balanced salt solution) in the NPS, a 2-fold dilution in PTF was chosen for the test. 50 µl of the diluted NPS was overlayed onto antigen coated plate as the primary antibody and direct ELISA was carried out as described in section 3.11.6 with RAHAP$_x$ as the secondary antibody. The optical densities obtained for antibody binding to HRSV antigen were corrected by subtraction of binding at equivalent dilutions to uninfected HeLa antigen and the results are shown in Figure 73. Mab1A12 was used together with GAMPx as a positive control and PTF was used in place of the primary antibody as a background control.

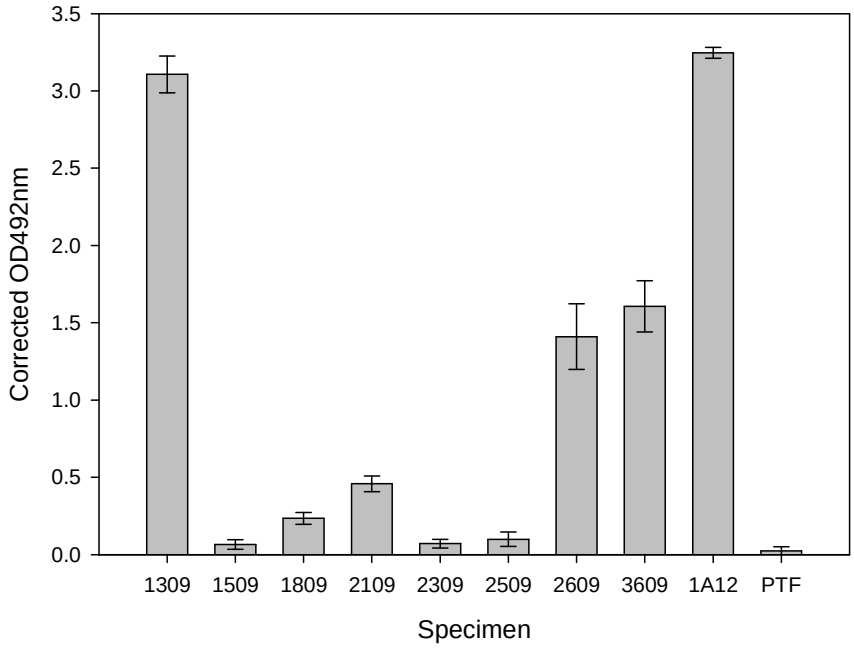


Figure 73 Immunoglobulin A in selected NPSs collected from 2008/2009 HRSV epidemic.

Among 8 selected NPSs screened for IgA, only one NPS (1309) yielded IgA titer comparable to Mab1A12. Two NPSs namely 2609 and 3609 yielded moderate IgA titer about half compared to Mab1A12. The rest of the NPSs only yielded mild IgA response with the corrected OD less than 0.5 with three of them (1509, 2309 and 2509) less than 0.1.

Thus NPS 1309 was selected as high IgA positive control, 2609 as medium IgA control and 2309 and 1509 as negative IgA control.

8.5 Specificity of RAHAPx

As IgA does not cross the placenta, the specificity of the anti-HRSV IgA assay in the presence of anti-HRSV IgG can be tested by assaying cord bloods simultaneously for IgA and IgG anti-HRSV antibodies. As IgA specific assay should prove negative despite the presence of high titer IgG maternal antibody in the cord blood.

Two Maxisorp plates were coated with HRSV and uninfected HeLa cell antigens as described in section 8.4. Sixteen cord bloods (see section 3.9.1) were diluted 1/40 in PTF, assayed in duplicate and used as the primary antibody. Direct ELISA was carried out with peroxidase conjugated anti-human IgG (RAHGPx) on one and peroxidase

conjugated anti-human IgA (RAHAPx) on the second plate as the secondary antibody. NPS1309 (1/2) (section 8.4), PTF and Mab1A12 was included as controls and treated the same as the cord blood except that the secondary antibody for Mab1A12 was replaced with GAMPx.

The optical densities obtained as a result of binding of antibodies to HRSV were corrected by subtracting the binding to the uninfected cell control and the results for both assays are summarised in Figure 74.

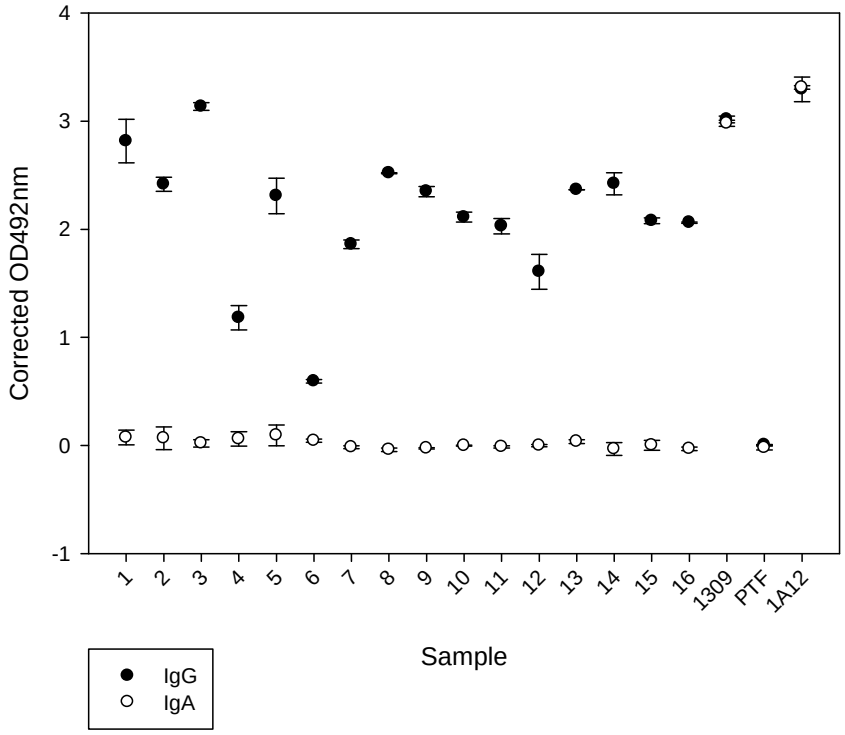


Figure 74 Direct ELISA on cord blood with RAHGPx and RAHAPx

All cord blood exhibited IgG reactivity with the mean corrected optical density between 0.5935 to 3.134 while IgA reactivity was indistinguishable from the background generated with PTF. NPS1309 gave a corrected optical density of about 3 with both RAHPx and PAHAPx showing the presence of both IgA and IgG at equivalent levels.

These results indicate that the RAHAPx based assay does not cross-react with IgG but is specific to IgA.

8.6 **Screening for IgA**

Maxisorp plates were coated, half with 50 µg/ml HRSV antigen and the other half with the equivalent concentration of uninfected HeLa antigen. Infants' sera were diluted 1/40 in PTF and assayed in triplicate and direct IgA ELISA was carried out as described in section 3.11.6 with RAHAPx as the secondary antibody. The IgA controls identified in section 8.4 were included at 2-fold dilutions in PTF and treated the same as the test sera. Primary antibody free PTF was included as a control for background activity of the peroxidase. 1A12 together with GAMPx as described in section 8.3 was included as a quality control for the HRSV antigen. The optical densities obtained for antibody binding to HRSV antigen were corrected by subtraction of binding at equivalent dilutions to uninfected HeLa antigen and the results are shown in Figure 75.

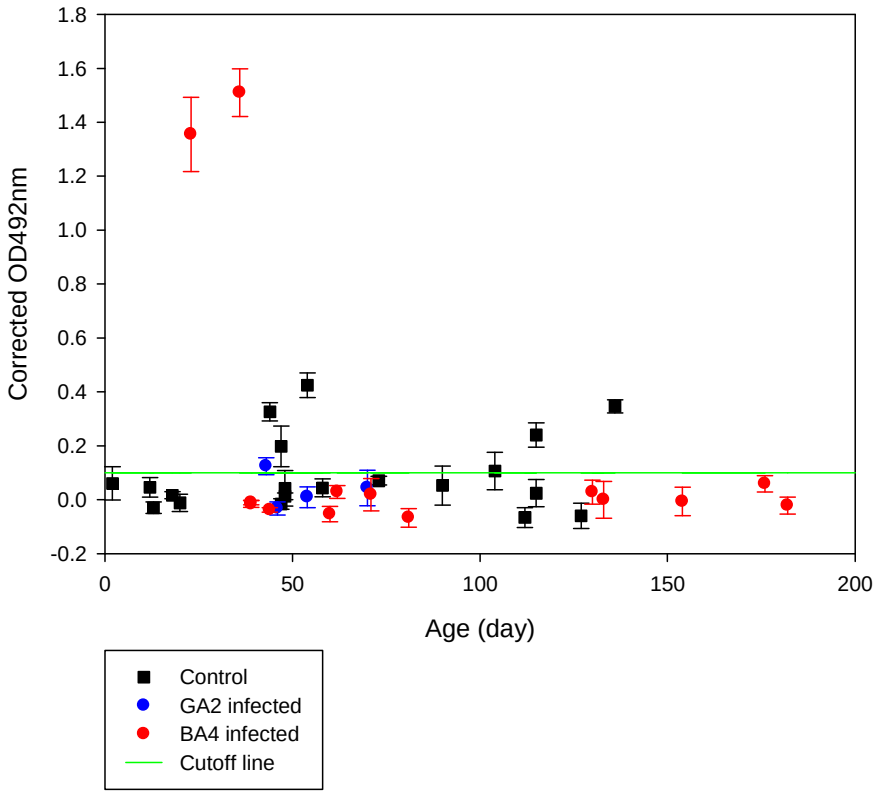


Figure 75 IgA titer against HRSV.

The corrected optical density for the IgA titer of the sera was plotted against the age when the specimen was taken as shown in Figure 75. Serum from infant volunteers infected with genotype GA2 and BA4 HRSV are depicted as blue dots and red dots respectively. Sera obtained from non-HRSV infected infant volunteers are depicted as black square boxes. The mean corrected optical densities ranged from -0.069 to 1.510. Negative values are due to higher optical densities to the uninfected HeLa cell control and regarded as zero. In a similar assay reported by Weaver et al.,(1991), a cut off value of >0.1 was chosen as indicative of prior HRSV infection and was adopted in this study. 30% (6 of 20) of the controls, 25% (1 of 4) of GA2 infected and 14% (2 of 14) of BA4 infected infants were concluded to have mounted an immune response to HRSV and were excluded from the study.

Chapter 9 Measurement of maternal antibody

9.1 Introduction

The measurement of maternal antibody to the G and F glycoproteins in infants' sera is described in this chapter. This is to test the hypothesis that genotype specific anti-G antibodies are protective and that infants hospitalised with severe HRSV infection lack genotype specific antibodies to the G glycoprotein of the infecting genotype.

HRSV infected infants under six months of age and admitted onto the children's wards of the Newcastle upon Tyne Royal Victoria Infirmary and Newcastle General Hospital were recruited between December 2009 and April 2010. Within 24 hours of recruitment a nasopharyngeal secretion and blood sample were collected from each infant. In order to allow a paired analysis of serum antibody levels, for each recruited HRSV infected, index child, it was initially proposed to recruit and collect serum from an aged-matched uninfected comparison infant, sampled in the same week. Recruitment of uninfected hospitalised infants within the target age group, however, proved more difficult than anticipated and matched pairs were not achieved. Only infants negative for serum anti-HRSV IgA antibodies as described in section 8.6 were entered into the study.

9.2 Measurement of antibody to GA2F

Sera were diluted 1/40 in PTF (section 6.2.4) for all ELISA assays. ConA was coated onto Maxisorp plates except for 18 wells reserved for controls. Half of each plate was loaded with GA2F antigen and the other half with an equal concentration of Bgal antigen. ELISA procedures were carried out in triplicate as described in section 3.11.7 with IgA free sera from infant volunteers (section 8.6) as the primary antibody and RAHGPx as the secondary antibody. To monitor the binding of ConA and antigens to Maxisorp plates, three ConA coated wells each were loaded with HRSV antigen, uninfected Hela control antigen, GA2F and Bgal control. These were stained with anti-HRSV Mab 1A12 followed by GAMPx as the primary and secondary antibody. These controls should give the corrected optical densities of >3.0. Six antigen free wells were loaded with PTF as the surrogate for the primary antibody and stained with RAHGPx and GAMPx to test the non-specific binding of the peroxidise conjugates to ConA.

The optical densities resulting from the binding of antibodies to GA2F (antibody ODs) were corrected by subtraction of the optical densities resulting from the binding to Bgal antigen at an equivalent concentration and the results are shown in Figure 76.

The coating of ConA on the Maxisorp plate and the capture of GA2F and HRSV antigens were optimal judging by the corrected optical densities of >3.0 as shown in Figure 77. There was no evidence suggesting non-specific binding of either of the peroxidase conjugates to ConA as both gave no measurable optical densities.

Due to the the absence of age matched comparison infants for the HRSV infected infants, a decay curve of the mean corrected antibody ODs of the comparison infant sera was fitted as described in section 3.22 using a non-linear regression model to predict the theoretical corrected antibody ODs of age matched comparison infants by extrapolation. The antibody ODs to the GA2F antigen showed an estimated half life of 40 days (Figure 76). For each HRSV infected infant the actual OD was compared with the predicted OD from the comparison infants decay curve for the child of that age and tested for significance by paired *t test*. All but three of the antibody ODs for the infected infants exceeded the predicted antibody ODs for an uninfected infant of their age . Overall the actual HRSV infected infant antibody ODs exceeded the predicted uninfected infant ODs by a mean of about 1.8 fold. This difference was statistically significant ($p<0.006$).

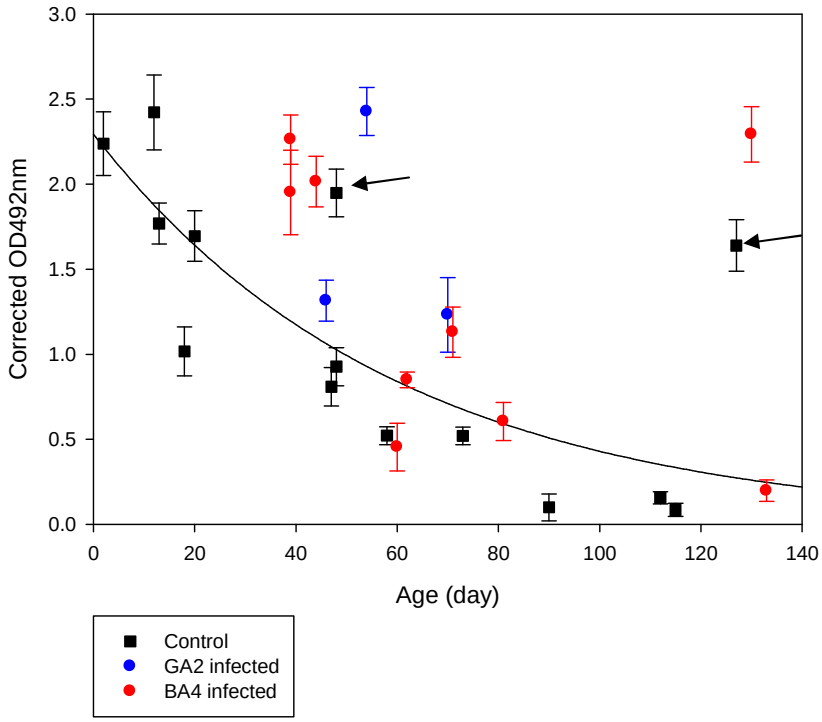


Figure 76 Maternal IgG to GA2F in infants plotted against their age.

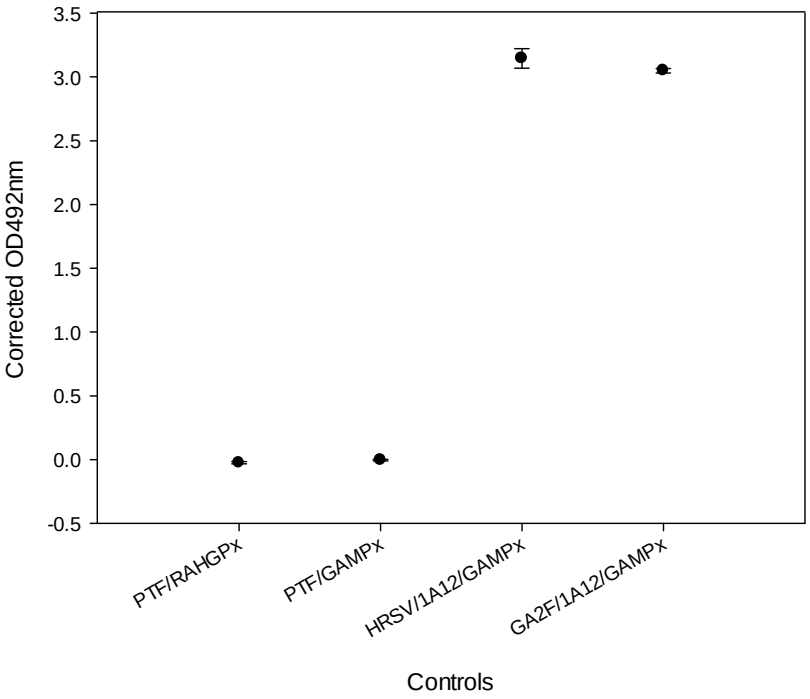
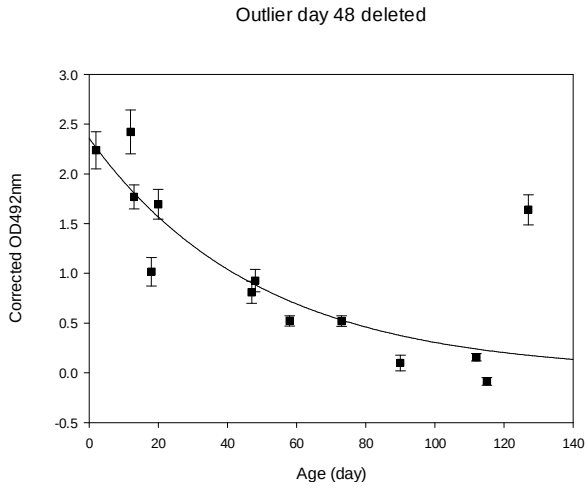
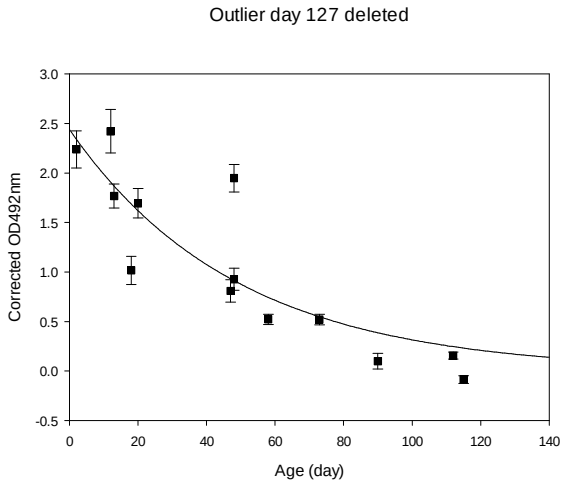


Figure 77 Corrected optical densities for the controls for the measurement of antibodies against GA2F

Two comparison infant outliers were present in Figure 76. To study the effect of outliers (see arrows in Figure 76) to the halflife of antibody ODs, a separate decay curve was generated with outlier day 48, 127 or both deleted (Figure 78). The deletion of outlier day 48, 127 and both altered the predicted antibody ODs at birth to 2.43, 2.44 and 2.50 respectively by extrapolation. Based on the antibody OD at birth, the deletion of outlier day 48, 127 and both reduce the halflife of the antibody ODs to 33, 34 and 28 days respectively. The latter is similar to the estimated halflife of the antibody ODs to the G glycoproteins estimated in sections 9.3 and 9.4 (below). A paired t test comparing the ODs every 20 days of age from birth to 120 days of age showed that the deletion of any one or both of the outliers did not produce a decay curve which is significantly different ($p>0.01$) from the decay curve without the omission of outliers. Thus, these outliers were not excluded from the analysis.





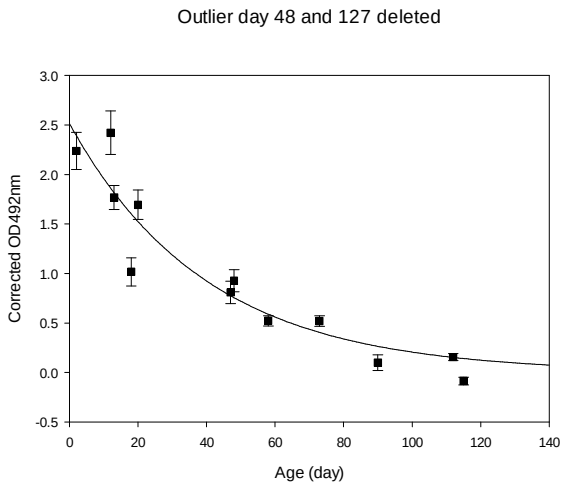


Figure 78 Effect of the deletion of outlier age day 48, 127 and both on the decay curve in the control sera.

9.3 **Measurement of antibodies to GA2G**

GA2G and Bgal antigens were captured onto ConA coated plate and ELISA was carried out as described in section 3.11.7 with infants sera (sections 8.6 and 9.2) and RAHGPx as the primary and secondary antibodies. Controls were similar to those used in section 9.2 but HRSV and GA2G and their corresponding control antigens were stained with Mab anti-HRSV G 133 and GAMPx as the primary antibody and secondary antibody respectively.

The optical densities resulting from binding of antibodies to GA2G (antibody ODs) were corrected by subtraction of the optical densities to Bgal antigen and the results are shown in Figure 79. The decay curve for the comparison group of infants was fitted as for GA2F in section 9.2 and is displayed in the same figure.

Neither conjugates showed any evidence of non-specific binding to ConA while HRSV and GA2G antigen showed optimal binding of ConA and antigens judging by the mean corrected ODs of 3.0 and 2.8 respectively (Figure 80).

The half-life of the antibody OD to GA2G in the comparison group was estimated to be 28.8 days from the decay curve. All three GA2 infected infants showed an antibody OD higher than the decay curve while 11/12 BAG infected infants showed antibody ODs higher than the decay curve despite not having been infected with a subgroup A virus. The mean antibody ODs of GA2G and BAG infected infants taken together were 1.4 fold higher than the mean predicted OD from the decay curve for children of the same age. Due to the low degrees of freedom from GA2 infected infants, the antibody ODs from both GA2 and BA4 infected infants were taken together and compared to the predicted antibody ODs for uninfected infants of the same age and the difference was statistically significant ($p=0.001$).

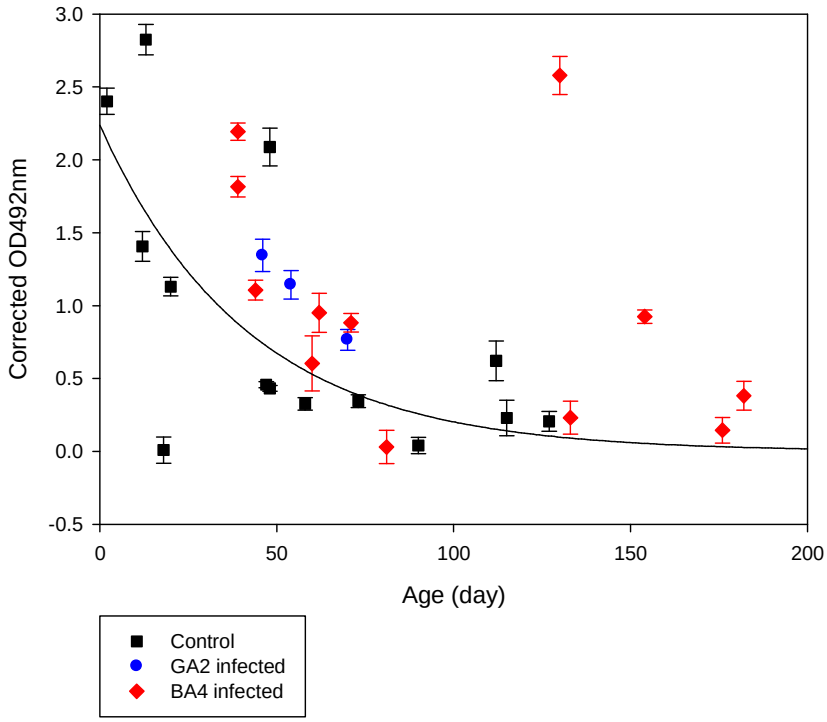


Figure 79 Maternal IgG to GA2G in infants plotted against their age.

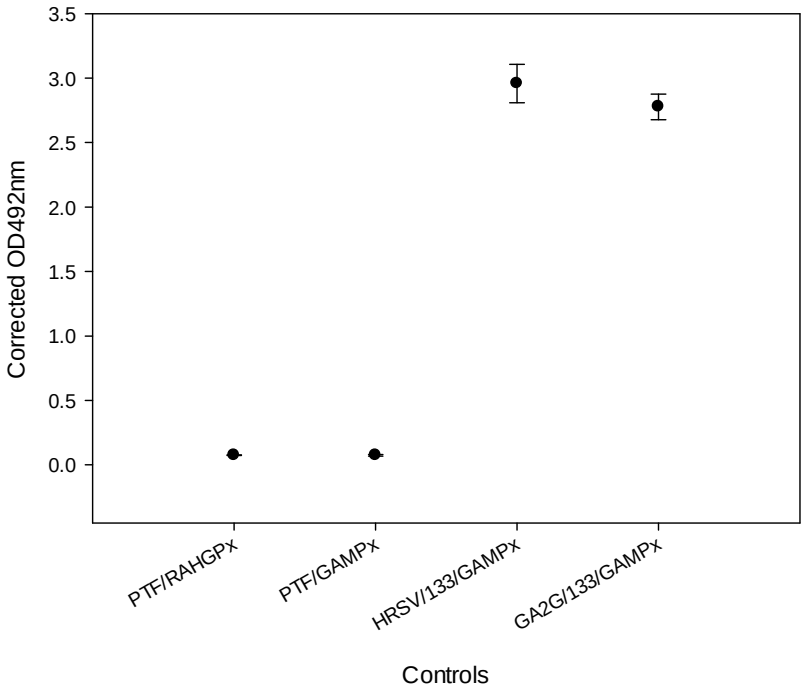


Figure 80 Corrected optical densities for the controls for the measurement of antibodies against GA2G

9.4 **Measurement of antibodies to BAG**

BAG and Bgal antigen was captured onto ConA coated plates and ELISA was carried out as described in section 9.3. The decay curve for the comparison group infants was fitted as described in section 3.22.

When the infant's sera were assayed on captured BAG as shown in Figure 81, very similar results were obtained as when assayed on captured GA2G as described above (Figure 79).

The conjugates did not show any evidence of non-specific binding while the antigen capture with HRSV and BAG yielded corrected optical densities of 3.1 and 3.0 showing optimal coating of ConA and efficient capture of antigens for the assay.

From the decay curve for the control sera the antibody OD to BAG decayed with a halflife of 33 days. All GA2 and 11/12 BA4 infected infants have serum antibodies to BAG higher than those predicted for uninfected infants of the same age by the decay curve. The mean antibody ODs for GA2 and BA4 infected infants taken together was on 1.5 fold higher than the predicted antibody ODs from the decay curve and these differences were statistically significant (p=0.0001).

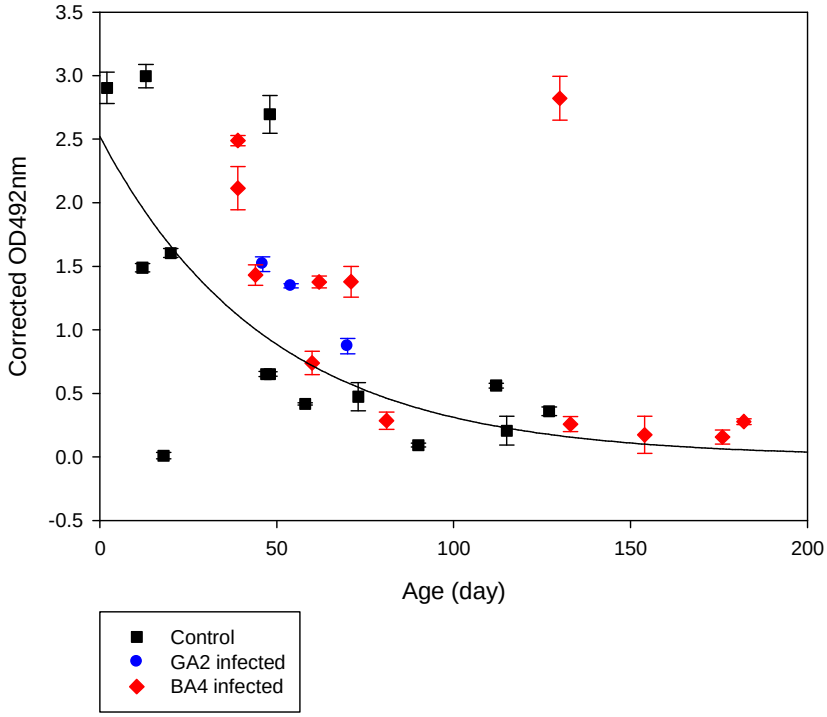


Figure 81 Maternal IgG to BAG in infants plotted againt their age.

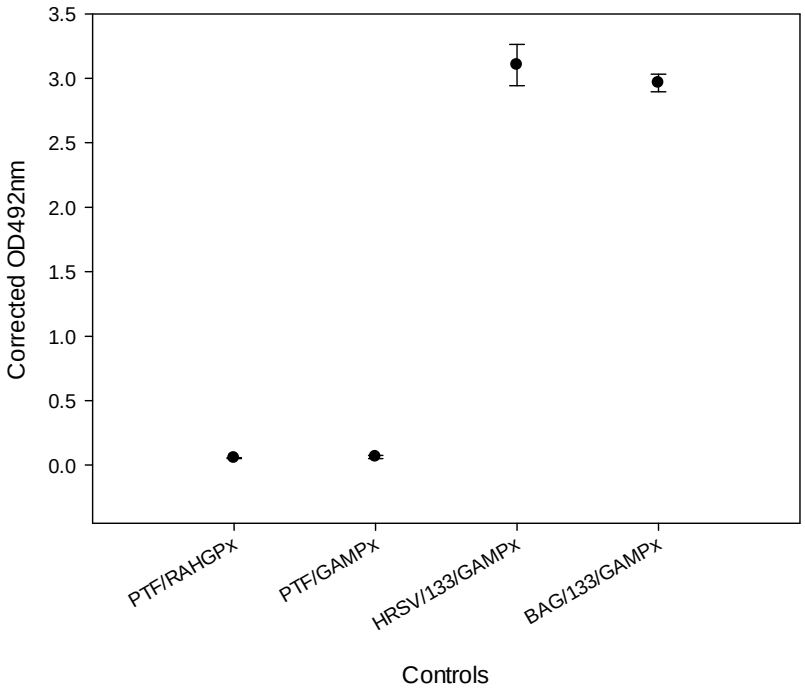


Figure 82 Corrected optical densities for the controls for the measurement of antibodies against BAG

9.5 **GA2G against BAG**

From Figure 79 and Figure 81 antibodies to the G glycoprotein of the homologous and heterologous subgroup seem similar. This was confirmed by plotting binding of antibodies to GA2G against binding to BAG for both index and comparison group and the results is shown in Figure 83. There was a highly significant linear correlation between the antibody ODs of GA2G and that of BAG (R^2 = 0.9725).

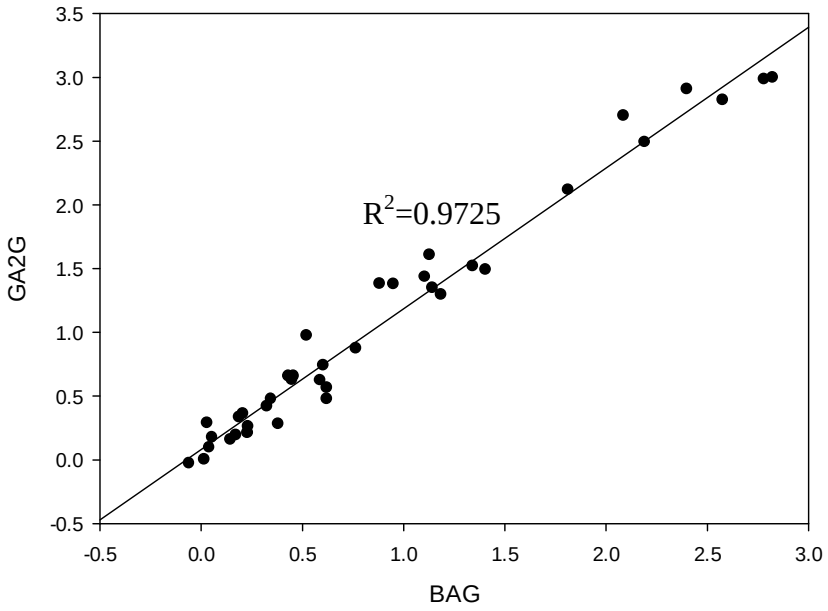


Figure 83 Antibody ODs of GA2G against BAG

The good correlation shows that the optical densities of anti-G maternal antibodies to both contemporary subgroups/genotypes were similar and there is no evidence here of a selective deficiency in antibody to either G lineage tested as initially hypothesised.

9.6 Age vs date of serum collection

It is not clear why antibody titres in the index group should be uniformly higher than the predicted antibody titres in uninfected infants of similar age. One possibility is that index and comparison groups are mis-matched. As HRSV circulates in the community predominantly in the winter months it is conceivable that antibody levels to a winter exposure may fall over the summer and autumn. This may affect the level of maternal antibody passed to an infant with babies born late in the year receiving lower levels of antibody than those born earlier. If this were the case, mismatch in the time of collection of index and control samples might introduce errors into the above analysis.

To test how the age distribution of volunteers varies with the date of collection, the age of both control and infected infants were individually plotted against the date of collection and displayed in Figure 84 and the R^2 values were calculated in Microsoft Excel 2007.

The age of control and infected infants recruited throughout the epidemic did not correlate with the date of collection, (R^2 values of <0.1) showing no significant bias of age with date of recruitment in either group. Never-the-less, early in the epidemic the index cases were mainly young infants. This would tend to bias the index cases to higher antibody levels than the decay curve for the controls. Further, 9 of 16 controls were collected after the last index case was collected (Figure 84). This would probably give a low estimate for the antibody levels in the control babies as they were collected further in time from the previous winter epidemic when the mother may have been infected. This mismatching of the comparison group may, at least in part, contribute to an underestimate of predicted antibody levels.

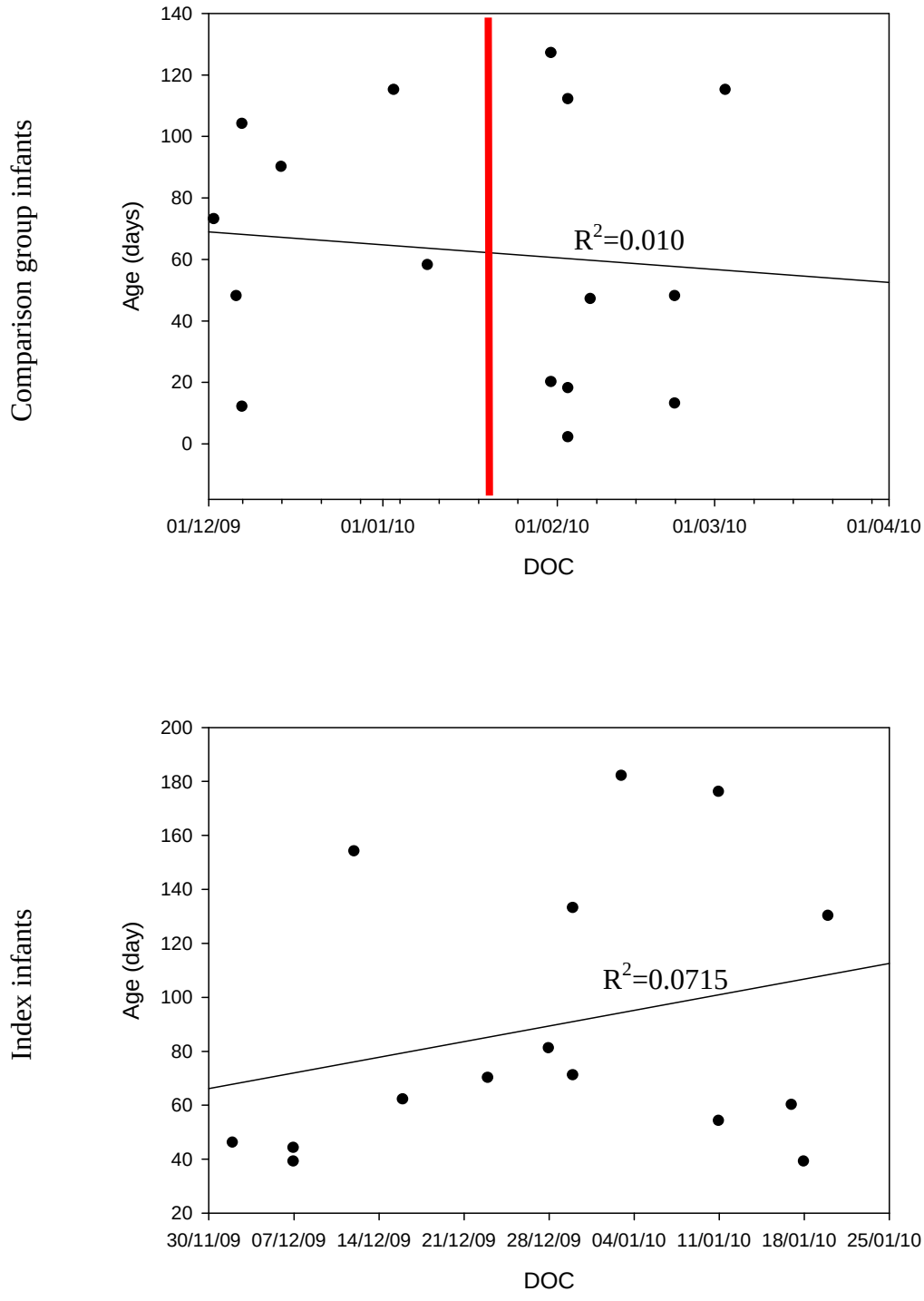


Figure 84 Age of infant against date of collection.

The red line in the control infants' scatterplot indicates the date where the last index infant was recruited.

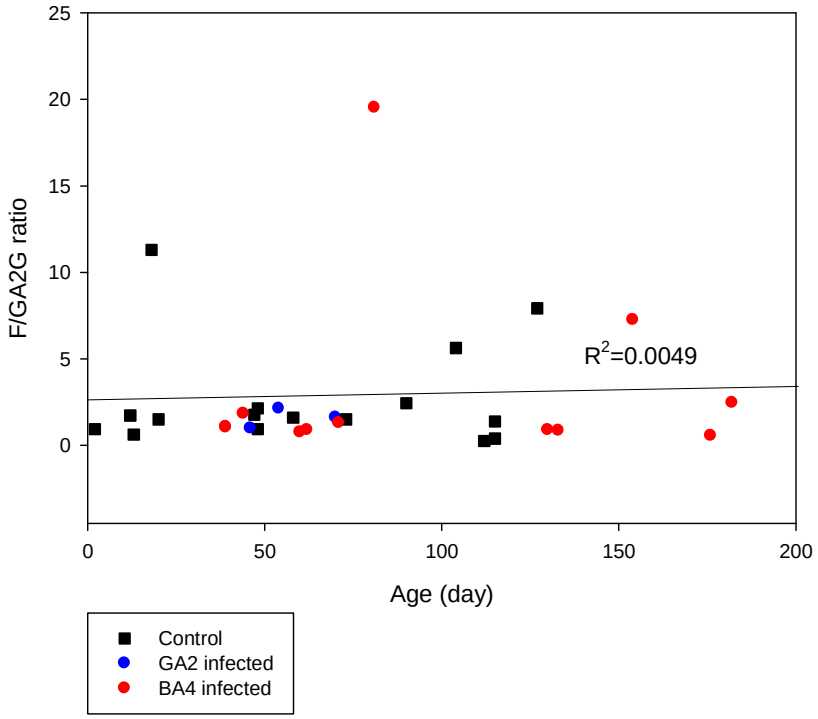
9.7 **F/G ratio**

As antibody levels in infant sera may vary not only as a result of differences in maternal immunity but with both age at collection and time of post-maternal exposure it is important to seek an assessment of anti-G antibody independent of the two latter variables. If it is assumed that antibody to the F glycoprotein will vary with regard to these two variables in parallel with anti-G antibody, the ratio of anti-F to anti-G should provide such a measure. As the F glycoprotein is largely conserved and antibody to the F glycoprotein, at least following primary infection, does not vary with the infecting strain (McGill *et al,* 2004b), a high anti-F:anti-G ratio measured on the infecting virus genotype will indicate a relative deficiency of genotype specific antibody to the infecting virus.

As demonstrated above, the half-lives of anti-F and anti-G antibodies in these infants were similar. To test if the the anti F/G ratio was independent of the age of the infant , the ratio of antibody ODs resulting from binding to the F glycoprotein to antibody ODs resulting from binding to the GA2G were plotted against the respective age of the infants. Only IgA free comparison group infants and infected infants were included and both groups were taken together for the analysis. This analysis was repeated for F/BAG and the representative scatterplots are shown in Figure 85.

As expected the F/G ratio did not correlate with the age of the infants (R^2 =0.005). Similar analysis was carried out by plotting the F/G ratios to the date of collection (Figure 86) it was found that the F/G ratio for both F/GA2G and F/BAG did not correlate with the time of specimen collection across the epidemic ($R^2 < 0.02$).

To test if the F/G ratio of the control group differs from the infected group, paired t test was performed on the F/BAG ratios. The results suggested that there were no significance difference between the F/G ratio of the control group and the infected group with the ($p<0.05$, 2-tailed). This does not support the hypothesis under test which predicts that the F/G ratio would be higher in the uninfected comparison group than in the infected group. Nevertheless, the sample size is small (n=15 for the infected group, n=13 for the comparison group).



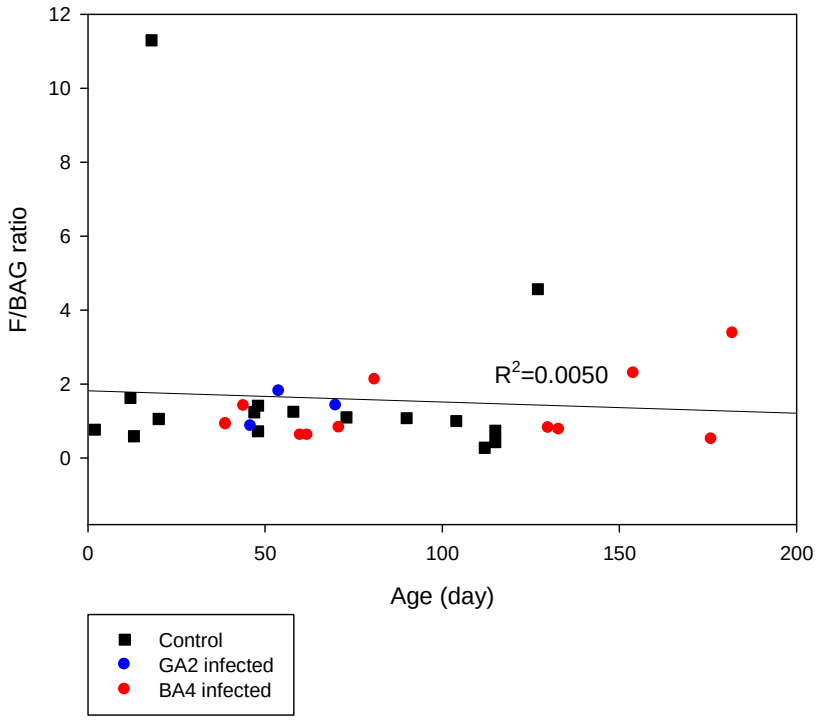
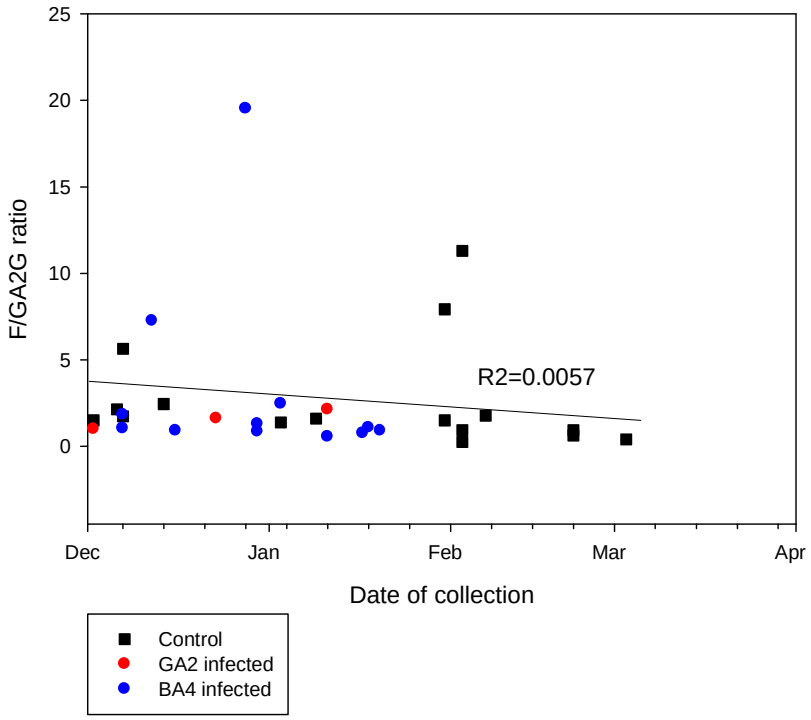


Figure 85 The antibody ODs of F/G ratio against the age of the infant volunteers.



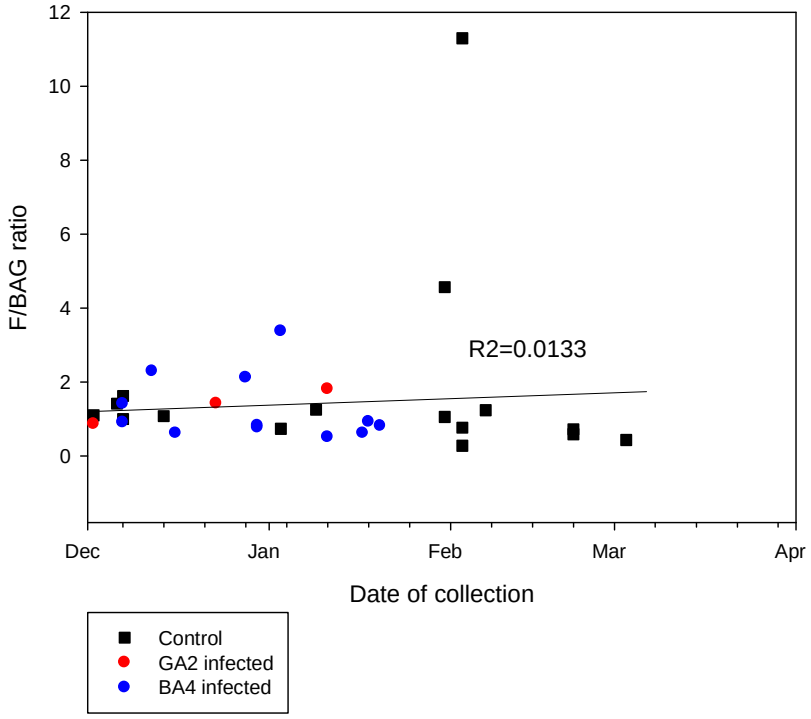


Figure 86 The antibody ODs of F/G ratio against the date of collection of the infant volunteers.

Chapter 10 Discussion

10.1 **Molecular epidemiology**

10.1.1 **Virus isolation**

The first aim of this project (Aim I) was to establish the molecular epidemiology of HRSV over the three consecutive epidemics studied, and to accurately define the contemporary genotypes. The first step was to obtain respiratory specimens in the form of NPSs and NSs for virus isolation in HeLa tubes.

The isolation rate for HRSV shows a reduction over the three seasons as shown in Figure 87. The interpretation of the rate of isolation must start with the specimen history. In the 2007/2008 and 2008/2009 epidemics, only NPSs positive by immunofluorescence to HRSV by the HPA, Newcastle were used for isolation. NPS are preferred over NS in the isolation of HRSV offering significantly increased sensitivity (Ahluwalia *et al.*, 1987; Masters *et al.*, 1987; T. Heikkinen *et al.*, 2002). This would explain the higher isolation rate for the first two epidemics compared to the third where mainly NS were collected (see Figure 87). The drop from first to second year remains unexplained. Although the isolation rate was high, at least in the first year, it did not achieve 100% probably for several reasons. Firstly, isolation was attempted only after the results of IF were made available by the HPA. This meant, in some cases, a delay of several days before inoculation of cell cultures during which time the specimen was held at 4°C. Secondly, specimens were stored at ambient temperature in the ward before being sent to the HPA for diagnosis bearing in mind that wards are well heated during winter. Thus the specimens spent a significant amount of time at a temperature range which is not conducive to the viability of HRSV. This does not take into consideration the amount of time the specimens were left at ambient temperature in the HPA before and after the diagnostic protocol was carried out before storage at 4°C. Finally, the specimens were diluted without any standard dilution factor. Low volume NPSs were often resuspended in HBSS to make up the volume for isolation.

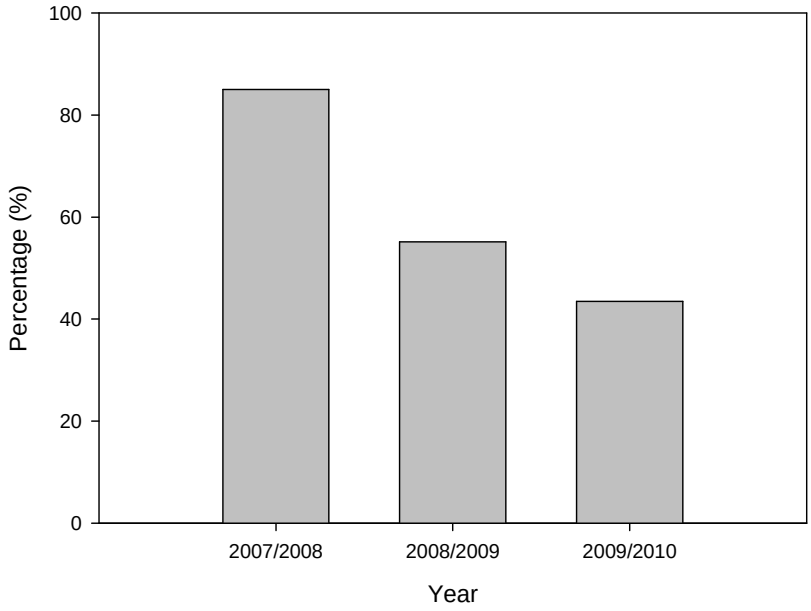


Figure 87 Isolation rate of HRSV over 3 epidemics

In the 2009/2010 epidemic, there was a major policy change in the HPA in dealing with specimens and in many cases, NPS were not readily available and freshly collected NS were used for virus isolation. The sensitivity of virus isolation is lesser with NS compared to NPS. However, the utilization of a more sensitive diagnostic assay can make up for the use of a less sensitive specimen (Meerhoff *et al.*, 2010) and approximately 60% of NS negative for HRSV isolation were positive by nested or heminested RT-PCR.

10.1.2 **Prevalence**

The RT-PCR amplification and sequencing of the C-terminal end of HRSV G gene and subsequent phylogenetic analysis has been widely used to determine the circulating virus genotypes (Patricia A. Cane *et al.*, 1992). Although the use of complete G sequence for the construction of phylogenetic trees can improve the bootstrap value the nodes and allow the definition of HRSV variants with different G gene length, analysis of partial gene sequences was chosen here for reasons of economy and speed. .

In this study revealed a predominance of subgroup A in the 2007/2008 and 2008/2009 epidemics while the subsequent epidemic was dominated by subgroup B HRSV. It was not surprising that subgroup A predominated for two of three epidemics in Newcastle as previous reports have shown the tendency of subgroup A to predominate more

frequently than subgroup B HRSV. For example, relatively recently subgroup A viruses predominated in 7 of 9 epidemics in Germany (Reiche and Schweiger, 2009), 5 of 8 epidemics in Belgium (Zlateva *et al.*, 2007) and 4 of 6 epidemics in Argentina (Viegas and Mistchenko, 2005).

The subsequent phylogenetic analysis revealed the appearance of three co-circulating genotypes namely GA2, GA5 and BA4. In the first two epidemics GA2 was the predominant genotype with GA5 and BA4 as the minor genotypes. The epidemic 2009/2010 showed a shift of the predominant group to BA4. In this epidemic, only GA2 co-circulates as the minor genotype while GA5 was not detected at all.

Neither GA2 nor GA5 are new in Newcastle upon Tyne having been previously reported in 1995-1997 by our group (A. McGill *et al.*, 2004a) although the number of representatives for genotype GA2 was low. A stable co-circulation of these two genotypes in the western European continent over many epidemics has previously been described. Co-circulation of GA2 and GA5 was reported in Germany from 1998-2007 with GA5 predominating in every epidemic (Reiche and Schweiger, 2009) and in Belgium from 1998-2006 where GA5 predominated in 7 of 10 epidemics (Zlateva *et al.*, 2007). Nevertheless, the prevalence of both GA2 and GA5 in Newcastle upon Tyne during the study period showed gradual decrease over time with the possible extinction of GA5 in 2009/2010. A similar phenomenon was reported in Ireland (Salter *et al.*, 2011), Edinburgh (Gaunt *et al.*, 2011) and Japan (Nakamura *et al.*, 2009; Goto-Sugai *et al.*, 2010; Fujitsuka *et al.*, 2011).

Previously identified genotypes of subgroup A in Newcastle upon Tyne (A. McGill *et al.*, 2004a) namely the GA1, GA3, GA7 and SAA1 were not detected over these three epidemics suggesting the absence from this continent although GA7 appeared in 1999/2000 and 2002/2003 in Germany (Reiche and Schweiger, 2009).

There was no other circulating subgroup B genotype detected over the three epidemics other than genotype BA which carries a distinctive 60 nucleotide insertion at the C terminus of the G gene (Trento *et al.*, 2003). This observation is similar to those reported in Argentina, India, South Afrika and Japan whereby all other prevailing genotype from subgroup B HRSV was completely replaced by BA viruses (Parveen *et al.*, 2006; Trento *et al.*, 2006; Nakamura *et al.*, 2009; Dapat *et al.*, 2010; Goto-Sugai *et al.*, 2010; Fujitsuka *et al.*, 2011; van Niekerk and Venter, 2011) suggesting an

undefined selective advantage of genotype BA over other subgroup B genotypes. However, genotype GB2 and GB3 were still detected in China during the 2008/2009 epidemic (Rong-Fang Zhang *et al.*, 2010a; Zhi-yong Zhang *et al.*, 2010b).

BA viruses were initially found to form four distinct clades namely BA1 to BA4 (Trento *et al.*, 2006) but 6 new clades namely BA5 to BA10 have recently been described (Dapat *et al.*, 2010). Genotype BA from Newcastle upon Tyne over the three consecutive epidemics was made up from a single clade BA4 which has been reported in Ireland, Edinburgh, Thailand, Argentina and Madrid where other earlier BA clades had been replaced by clade BA4 (Boonyasuppayakorn *et al.*, 2010; Trento *et al.*, 2010; Salter *et al.*, 2011).

10.2 **Recombinant protein expression**

Sera for assessing anti-G antibody levels were collected from infants only in the 2009/2010 epidemic when the prevalent genotypes were GA2 and BA4. In order to develop an ELISA assay to measure the maternal antibodies to the G glycoproteins in these sera (refer to Aim II), the G glycoproteins of representative strains of both genotypes GA2 and BA4 were cloned and expressed in MVA under the control of the vaccinia virus p7.5 promoter. In order to measure antibodies to the F glycoprotein, as the F glycoprotein is largely antigenically conserved, the F gene of only the GA2 strain was expressed likewise.

Recombinant F and/or G glycoproteins of HRSV have been successfully expressed in the vaccinia virus expression system to high level and the expressed glycoproteins were shown to be highly immunogenic and antigenic in rodents (Elango *et al.*, 1986; R A Olmsted *et al.*, 1986; Stott *et al.*, 1986; G W Wertz *et al.*, 1987), monkeys (Robert A. Olmsted *et al.*, 1988) and chimpanzees (Crowe *et al.*, 1993). The immunization of mice with recombinant G glycoprotein induces virus specific CD4+ T cell responses which involve both Th1 and Th2 (Teresa R. Johnson *et al.*, 1998; Varga *et al.*, 2000) and confers protection in the lungs shown by a significant reduction in the virus titers following subsequent intranasal challenge with the homologous HRSV strain (Wayne M. Sullender *et al.*, 1990), a phenomenon which was also demonstrated in chimpanzees (Peter L. Collins *et al.*, 1990). Immunization of cotton rats with recombinant F glycoproteins also induced high levels of serum neutralizing antibodies which was 2-3 fold higher compared to those induced by HRSV infection (R A Olmsted *et al.*, 1986). Recombinant F and G glycoproteins expressed in MVA was shown to be highly

immunogenic in mice (Wyatt *et al.*, 2000) and protected mice from HRSV challenge post immunization (Olszewska *et al.*, 2004) but experimental work in macaques failed to show any protection upon challenge with HRSV four months after vaccination (de Waal *et al.*, 2004).

The recombinant F and G glycoproteins expressed from recombinant vaccinia virus were shown to be indistinguishable from the authentic HRSV glycoproteins in terms of glycosylation, disulphide linkage, electrophoretic mobility, cell surface expression and antigenic specificity (Ball *et al.*, 1986; Elango *et al.*, 1986; R A Olmsted *et al.*, 1986). Further characterization of the expressed G and F via western blotting would definitely be helpful in providing valuable information regarding the molecular weight and the glycosylation of the respective glycoproteins.

Interestingly, in the current study the expression of the recombinant fusion glycoprotein of HRSV genotype GA2 in MVA showed rapid syncytia formation in HeLa cells, a phenomenon not seen during expression in CV-1 and Hep-2 cells of fusion glycoprotein of prototype strain A2 in WR vaccinia virus although in both cases expression was controlled under the p7.5 vaccinia virus promoter (R A Olmsted *et al.*, 1986). This apparent discrepancy may be attributed to the masking effect of the vigorous CPE on the cells of vaccinia virus infection compared to the more attenuated MVA. The formation of syncytia by recombinant HRSV fusion glycoprotein was observed in recombinant vesicular somatitis virus propagated in BHK cells (Kahn *et al.*, 1999) and Simian virus 40 (SV40)/monkey cells (R A Olmsted *et al.*, 1986). However, none have reported the observation of syncytia as rapid as 1 hour p.i. Furthermore, other authors expressing recombinant F glycoproteins in MVA did not report the occurrence of syncytia in cell culture (Wyatt *et al.*, 2000; Olszewska *et al.*, 2004).

F glycoprotein expression was regulated by the p7.5 promoter, which is active throughout the life cycle of MVA. The early stage genes of vaccinia virus in HeLa cells can be detected 20 min p.i. and peak around 100 min p.i. before declining with the half life of 30 min (Baldick and Moss, 1993). Commensurate with this F glycoprotein, although undetectable immediately after inoculation, was demonstrable in cells infected with recombinant MVA one hour post infection supporting the observation that the formation of syncytia at approximately 1 hour p.i. is attributable to the newly synthesised recombinant F.

10.3 **Concanavalin A captured ELISA**

The ConA capture ELISA was adopted to capture the expressed fusion and attachment glycoproteins without prior purification (Aim III). The capture of the F and G glycoproteins of HRSV strain A2 expressed in vaccinia virus under the control of bacteriophage T7 RNA polymerase promoter has previously been reported (M. J. Robinson, 2007). Coating of ConA onto Maxisorp plates at high pH in BCB in the latter study was found to be suboptimal (section 6.2.3) and this problem was solved by coating ConA at physiological pH.

Each subunit of ConA binds one molecule of saccharide with the greatest affinity to the non-reducing ends of α-linked mannose residues and their glycosides in α-anomeric form (Goldstein *et al.*, 1965). ConA exists in three forms namely monomeric, dimeric and tetrameric. The monomeric form is approximately 26-27kDa and forms a dimer at pH5 (Abe *et al.*, 1971), a tetramer at physiological pH and undergoes irreversible denaturation at pH above 8 at room temperature (Pflumm *et al.*, 1971). The ability to bind saccharides reduces from tetrameric>dimeric> monomeric form (Wang and Edelman, 1978; Ishii *et al.*, 1984). Thus, the coating of ConA at pH 9.6 would be expected to have a detrimental effect on ConA binding while coating at pH7.4 allows the formation of the most potent tetrameric form.

10.4 **Epitope mapping**

Having established a method suitable for the assay of antibodies to the individual glycoproteins of the infecting virus strains it remained to attempt differentiation of antibody to the conserved G epitopes from variable epitopes (Aim III). To facilitate the design of a serologic tool for the measurement of maternal antibodies directed against conserved epitopes it was decided to map the epitopes recognised by the cross-reactive monoclonal antibodies 133 and 21 in the hope that an immunogen capable of recognising exclusively cross-reactive antibodies could be constructed. .

The antigenic map of the G glycoprotein has been previously mapped, by competitive ELISA with 19 Mabs raised against the Long strain, into 9 antigenic areas namely antigenic areas I to IX with area VI, VII, VIII and IX showing the greatest degree of overlap (B Garcia-Barreno *et al.*, 1989). Further work using another set of Mabs raised against HRSV strain Mon/3/88 tested on subgroup A and B HRSV by western blot revealed three distinct patterns. Two Mabs, which reacted with both subgroups A and B,

identified conserved epitopes of the G glycoprotein. Seven Mabs reacted with only subgroup A but not subgroup B viruses and identified subgroup A specific epitopes. The remaining ten Mabs reacted with some viruses from subgroup A but not other strains from a different genotype and identified strain specific epitopes (I. Martinez *et al.*, 1997). Walsh and co-workers (1989) were also able to differentiate 5 subgroup A specific epitopes, 11 subgroup B specific epitopes and 3 conserved epitopes. Two of their three Mabs to the conserved epitope namely K6 and L9 have been further mapped within residue aa162-172 and 151-172 respectively (Murata *et al.*, 2010). These observations suggest that the epitopes of specific antigenicity clusters within certain defined regions which can be defined and that the conserved epitopes overlap the conserved 13 amino acid sequence between residues 164 and 177.

The initial assumption that the Mabs to the conserved epitope (Mab133 and Mab21) would bind the highly conserved 13aa sequence was not supported when tested by peptide ELISA. It appears that these antibodies either bind to shorter runs of conserved amino acids embedded within the variable regions of the molecule or that binding to the conserved central region is dependent upon interaction of that region with other regions of the polypeptide chain or the sugar molecules, which make up the bulk of the mass of the mature glucoprotein. Palomo et al., (1991) have shown that epitope binding of most G specific Mabs are glycosylation dependent binding strongly to the mature G glycoprotein but to a lesser extend the O-glycosylated species and the unglycosylated precursor. One of their Mabs bound to N-glycosylated intermediates but not an O-glycosylated intermediates suggesting an unique glycosylation requirement for each epitope. In addition to this (Rawling and Melero, 2007) have shown that anti-lectin and carbohydrate Mabs are common and have been used extensively to study the glycosylation of proteins.

To study the effect of glycosylation on the binding of the Mabs to the conserved epitope, a glycosylation inhibitor, tunicamycin was used to inhibit N-glycosylation of HRSV propagated in HeLa cells. Tunicamycin enters the HRSV infected cells via a putative plasma membrane transporter known as MFSD2A (Reiling *et al.*, 2011) and inhibits the first step in the lipid linked oligosaccharide pathway which produces GlcNAc-pyrophosphoryldolichol (Heifetz *et al.*, 1979) essential in the addition of N-linked sugar side chains. The effect of tunicamycin is not efficient as its effect is dose dependent and it also induces endoplasmic reticulum (ER) stress which leads to cell toxicity and death (Reiling *et al.*, 2011) which can be observed as the rounding up of cells under a

microscope. Tunicamycin treated HRSV infected cells yielded three extra bands not found in tunicamycin free virus culture The size of the bands was approximately 36, 33 and 32 kDa. The 36 kDa band corresponded to the electrophoretic mobility of unglycosylated Gm in SDS PAGE (Gail W. Wertz *et al.*, 1985; Roberts *et al.*, 1994). Although the predicted length of unglycosylated Gm is 32kDa. the high proline content is thought to contribute to the slower electrophoretic mobility in SDS-PAGE (G. W. Wertz *et al.*, 1989). This suggests that tunicamycin induced ER stress also affects O-glycosylation in the Golgi apparatus. The 32kDa band corresponded to the electrophoretic mobility of Gs (Roberts *et al.*, 1994). The 33kDa band probably corresponded to the translational product from the second AUG start codon at Met48. Visualization of these bands by MAbs 133 and 21 in Western blots suggests that the binding of Mab133 and Mab21 is not dependent on either O-glycosylation nor N-glycosylation and that the antibodies bind the peptide backbone of the G glycoprotein.

Further mapping of the binding sites of two cross-reactive (Mab133 and 21) together with two A2 strain specific (Mab4G4 and 3F43) and one subgroup A specific monoclonal antibodies (Mab1C2) was attempted. The first method of mapping attempted involved additive binding ELISA to study the relationship of the epitopes of each monoclonal antibody. Previously the mapping of epitopes on the G glycoprotein was conducted by a similar method using competitive ELISA (Palomo *et al.*, 1991) which requires the labelling of the monoclonal with a large reporter protein such as biotin which can potentially alter the binding of some Mabs (Edward E. Walsh *et al.*, 1989). Palomo et al, (1991) have shown that sera from covalescent human failed to block the binding of the majority of the murine monoclonal antibodies in competive ELISA suggesting that the immunodominant epitopes involved in the murine system might be irrelevant in the natural infection in humans. Walsh et al., (1989) on the other hand carried out competitive ELISA with murine Mabs with and without conjugation with biotin as a reporter molecule and found out that biotinylation changed the binding of some of the Mabs while three Mabs failed to be biotinylated. Similar inactivation of antibodies by peroxidase labelling has also been reported (B Garcia-Barreno *et al.*, 1989). In additive binding ELISA the antibodies are unlabeled retaining their native specificity and avidity for the corresponding epitope. The results of the additive binding ELISA presented here suggest that the conserved epitopes recognised by Mab133 and Mab21 cluster closely to both subgroup specific and strain specific epitope recognised

by Mab1C2 and Mab4G4 respectively but are distinct from the strain specific epitope of Mab3F43.

In the second method of epitope mapping the G gene of HSRV GA7 was progressively truncated from both -3' and -5' end and the truncated fragments were transiently expressed in HeLa cells and screened by immunofluorescence assay to refine the location of the epitopes.

Using this approach the epitope of Mab1C2 (subgroup A specific) was mapped within residue aa158-190. This agrees with the previous observation of Mekseepralard et al., (2006a) who demonstrated that this Mab bound to a BSA conjugated peptide corresponding to aa172-187, a subgroup A conserved epitope which overlaps the N-terminus of the wholley conserved region of the G polypeptide. The vaccination of peptide corresponding to aa174-187, a subgroup A conserved epitope, induced protective immunity in rodents when challenged with HRSV from the same subgroup but the antibody to this epitope is non-neutralizing *in vitro* (Trudel *et al.*, 1991; Simard *et al.*, 1997). In addition to this, Mab1C2 also confers passive immunity when administered prophylactically (Mekseepralard *et al.*, 2006b).

MAb 4G4 has previously been reported as strain specific for the sub-group A2 strain of HRSV (Morgan et al., (1987). However, the reactivity of Mab4G4 to GA7G in this study contradicts that finding. The authors screened Mab4G4 on untyped HRSV strains isolated from 1965-1986 by western blot which may not have included the GA7 genotype. The results presented here also contradict McGill et al., (2005) who showed no binding of Mab4G4 to HRSV genotype GA7 in a Biacore assay. The authors used strain 17063 compared to 25173 (refer to Figure 22) used in my experiment but these two strains clustered together within the GA7 clade supported with a bootstrap value of 99%. However, the 99% identity refers to the C-terminus end distal to the binding site of Mab4G4 which is within the central unglycosylated region. It appears, therefore, that 4G4 binds to only a restricted number of sub-group A strains. The epitope for Mab4G4 is currently unknown but here mapped within aa155-173. Within this sequence, N-terminal residues 164 to 173 resides are the 13 aa conserved sequence and their removal ablated binding. The only variable residue in the 4G4 epitope capable of conferring strain specificity is residue 157 which is either a serine or asparagines. The alignment of aa155-177 (Table 22) of strain A2 and GA7 revealed that amino acid at position 157 is a serine and asparagine respectively. Both serine and asparagines are amino acids with

polar uncharged side chains and probably the differences in experimental setting have contributed to these observations.

Table 22 Amino acid sequence alignment between nucleotide 155-177 of strain A2 and GA7

Genotype (Strain)	Amino acid sequence (155-177)
GA1 (A2)	PP**S**KPNNDFHFEVFNFVPCSIC
GA7 (25173)	PP**N**KPNNDFHFEVFNFVPCSIC

Amino acid 157 is in bold and underlined.

The truncation experiments suggest that the epitopes of Mab133 and Mab21 map to aa155-190 within the unglycosylated central core. The deletion of residues 155-158 ablated binding of Mab133 and severely reduced binding of Mab21 suggesting that at least one of these residues contribute to an epitope. The observation that 21 but not 133 showed low level binding to the 158-284 truncate suggested that there may be some differences between the epitopes of the two Mabs. Residue 155 and 156 but not 157 are likely to the part of the epitope for 133 because both residue 155 and 156 are both prolines which are conserved between subgroups but 157 can be either serine or asparagine in subgroup A and is lysine in subgroup B. Deletion of residues 1-177 from the C-terminal also ablated the binding of both Mabs showing that residue 177-190 is also required for binding. This indicates that these Mabs recognise a complex epitope in which these two disparate parts of the polypeptide chain are brought together.

Thus conserved epitopes overlap with the both strain and subgroup specific epitopes suggesting that the distribution of the conserved, subgroup specific and strain specific epitopes within the central unglycosylated region are not distinct entities within the polypeptide chain as initially suggested by Martinez et al., (1997). This observation precludes the manufacture of a synthetic antigen corresponding specifically to the conserved epitopes of G and failing to react with either subgroup cross-reactive or strain specific antibodies which was the aim of this project (Aim III).

10.5 **Volunteers recruitment**

The study of the protective role of maternal antibodies in infants against HRSV has previously been investigated by the comparison of an infected index group against an uninfected comparison group. Researchers have used different criteria for recruitment into the comparison group. For example, Brandenburg et al., (1997) have compared 38 children hospitalized with HRSV disease as the index group with a control group consisting of 45 children participating in a Hepatitis B virus vaccination trial which was recruited off epidemic to minimize the possibility of HRSV infection. Gimenez et al., (1996) compared infants hospitalized with HRSV disease against age and sex matched infants either without HRSV infection or protected against severe disease. In the current study, two groups of volunteers were recruited (Aim IV). The index group comprising infants of less than six months of age with severe HRSV infection requiring hospitalization and a comparison group attending outpatient clinics or hospitalized due to reasons other than respiratory disease and with no evidence of prior infection with HRSV. Ideally, each index case would be matched for at least one aged and sex matched comparison group infant sampled on the same day. Reality fell far short of this ideal. Insufficient comparison group infants were identified and pair wise analysis was not possible.

10.6 **IgA screening**

All sera were screened for IgA as an indicator for host's humoral response as maternal IgG is indistinguishable from infant's IgG other than avidity (Meurman *et al.*, 1992). Thus in order to eliminate the probability of measuring infant's IgG from a prior HRSV infection, volunteers must be screened for anti-HRSV IgA which does not cross the placenta. The presence of serum IgA can result from a genuine humoral response to an HRSV infection or from passive uptake of IgA from colostrums (Weaver *et al.*, 1991). Although colostral IgA is largely dimeric and serum IgA is largely but not entirely monomeric, no literature to date had described methods to differentiate colostral IgA from serum IgA. However, Weaver et al., (Weaver *et al.*, 1991) report detection of peak titers of anti-HRSV antibody in colostrum fed infants of only 1/16 whilst IgA screening in the present study was carried out on sera diluted 1/40.

The measurement of total IgA to whole HRSV antigen is likely to be sensitive as anti-HRSV serum IgA (maternal) has been shown to recognise at least the conserved fusion glycoprotein and only to a lesser extend the variable G glycoprotein (Yamazaki *et al.*,

1994). Whilst Brandenburg et al., (1997) have reported a lower IgA and IgM positive percentage of 41%. Roca et al., (2003) found that 78% of infants infected with RSV made a detectable IgA antibody response 1 month after infection. The latter authors similarly excluded anti-HRSV IgA positive infants as determined by a membrane fluorescence test, from a study of maternal antibody levels. The incidence of prior infection in control infants was 12% lower than that in the current study (30%), therefore, there is still a possibility that monitoring anti-viral IgA will miss some prior infections. This may account for the infants with exceptionally high IgG antibodies which fall as outliers on the decay curve for the comparison group (refer figure 76, 79 and 81).

10.7 **Analysis**

The infants from both groups should ideally be of the same age at the time of recruitment. However, the lack of volunteers did not permit for this ideal to be achieved. Thus, two approaches to data analysis were adopted. Firstly, the decay curves for anti-F and anti-G antibody in the comparison group were plotted.

10.8 **Antibody decay**

To study the antibody decay, scatterplots of the antibody ODs against the age of the control group were plotted and it was observed that younger infants have higher antibody compared to the older infants and the scatterplots mimic the exponential decrease shape. A non-linear regression curve was then fitted using the exponential decay algorithm as previously applied by Allansmith et al., (1968) and Cloonan et al., (1970). The non-linear regression model described here is comparable with the linear regression model on a logarithmic scale used by other authors (Cox *et al.*, 1998; Ochola *et al.*, 2009).

The maternal antibodies to the F and G glycoproteins of HRSV in IgA response negative comparison infants measured as the antibody ODs show half lives of about 40 days and 28.8-33 days respectively. The omission of outliers reduces the half life of antibody to the fusion glycoprotein to a mean of 28 days. The half life reported here is closer to 21 to 26 days as reported by Brandenburg et al., (1997) and reviewed in Collins and Crowe (2001) which suggested that the half life of antibodies to the individual glycoproteins are similar to the half life of total antibodies to the whole HRSV. However, a much longer mean maternal antibody half life of 79 to 99 days have

been reported (Ward *et al.*, 1983; Cox *et al.*, 1998; Ochola *et al.*, 2009). The mean half life of the passively acquired maternal antibodies to other viruses ranges from 21 to 80 days (refer to Table 23) and high variation of half life within (Cloonan *et al.*, 1970) and between studies has been reported (Caceres *et al.*, 2000; Shilpi *et al.*, 2009). The titers of measles specific maternal antibodies present in German newborns were reported to be two-fold higher than in Nigerian newborns (Hartter *et al.*, 2000) suggesting that the transfer of maternal antibodies from mother to foetus might be ethnic, demographic or continent dependant so that the half life of maternal antibodies of studies conducted from countries to countries or among different ethnic groups within countries should be interpreted with care.

Table 23 The half-life of passively acquired maternal antibodies to various viruses in infants.

N	Country	Virus	Method	MatAb half-life	References
100	Thailand	Dengue	HI and PRNT50	41	(Watanaveeradej *et al.*, 2003)
69	Itali	Influenza H1N1	HI	84.4	(Zuccotti *et al.*, 2010)
120	Australia	Rubella	HI	43 (14-259)	(Cloonan *et al.*, 1970)
88	UK	Polio	PRNT50	21	(Perkins *et al.*, 1958)
66	USA	Polio	PRNT50	25	(Gelfand *et al.*, 1960)
34	USA	Measles	PRNT50/HI	46.1-60.8	Reviewed in (Caceres *et al.*, 2000)
47	Bangladesh	Measles	ELISA	35-45	(Shilpi *et al.*, 2009)
27	Netherlands	Varicella -Zoster	ELISA	25.5 (14.6-76.0)	(van der Zwet *et al.*, 2002)
66	USA	hPIV3	HI	51 (42-60)	(Min-Shi Lee *et al.*, 2001)

HI=Heamagglutination Assay, PRNT50=Plaque reduction neutralization assay 50

The antibody ODs to the F and G glycoproteins of HRSV were found to be significantly higher than the predicted antibody ODs obtained from the corresponding decay curve for the control group. This might mean that these HRSV infected infants might have mounted their own humoral response against the infecting virus strain by the time of testing. Against this, however, it has previously been observed that primary infections engender genotype specific anti-G antibody responses (A. McGill *et al.*, 2004b). If the excess of anti-G antibodies were due to responses to the primary infection of the infant they would be expected to be type specific for the infecting genotype. This however was

not the case here as the anti-BA antibodies to both BAG and GA2G were higher than predicted in infants infected with the BA virus.

The higher antibody ODs of infected infants to the predicted age matched control might be taken to indicate that deficiency in strain specific anti-G glycoprotein is not a risk factor for the pathogenesis of severe HRSV disease in infants which contradicts the findings of other researchers who have found positive correlation of high HRSV specific maternal antibodies to protection in infants (Glezen *et al.,* 1981a; Ogilvie *et al.,* 1981; Kasel *et al.,* 1987).

The higher levels of antibody in the index group are, thus, a surprise and may indicate that the comparison and index group were not truly comparable. There is some evidence that anti-HRSV maternal antibody may fall across the epidemic (Nandapalan *et al.,* 1986). A lower mean antibody titer in the control group might be produced, therefore, if comparison infants were recruited later in the epidemic than the index group. This proved to be the case and it was further noted that the younger index cases tended to be recruited earlier in the epidemic. As antibody titers decay exponentially this would tend to raise the mean antibody titers of the index group.

It is noteworthy that Brandenburg et al., (1997) have used a comparison group recruited during off epidemic period about 2-3 years before the recruitment of index group while Gimenez et al., (1996) measured HRSV specific antibody from the cord blood while assuming that the decay of maternal antibody was a constant as determined by Cloonan et al., (1970). As the level of antibody in infant sera is likely to vary with the level of immunity of the mother, which may vary with the time of birth post-exposure of the mother, and the age of the infant, direct comparisons of mean anti-G glycoprotein antibody levels in index and comparison groups are unreliable as a test of the hypothesis that the index group are anti-G antibody deficient. However, essentially all mothers transfer high levels of strain cross-reactive anti-F glycoprotein antibodies to the infants (A. McGill *et al.,* 2004b). The hypothesis predicts that index infants will be deficient in anti-G antibodies specific to the infecting strain. Such a deficiency would be apparent as a reduced ratio of anti-G to anti-F antibodies in the index group. This approach depends on the assumption that both anti-G and anti-F antibodies decline in infants and in their mothers at a similar rate. It has only been possible here to demonstrate approximately equal decay rates of anti-F and anti-G antibodies in infants (see above). Thus the ratio of anti-G and anti-F antibodies were compared. Anti-F/anti-G ratios were shown to be

independent of the age of the infant allowing direct comparison of their means in index and comparison groups. No significant differences were observed between anti-F/anti-G ratios in index and comparison groups suggesting that hospitalized children are not selectively difficient in anti-G antibodies to the infecting virus strain as the hypothesis postulates.

This failure to find any evidence of a deficiency in anti-G antibodies in infected infants supports the findings of Vieira et al. (2007). These authors measured the subtype specific antibodies, presumably of maternal origin, to the subtype specific antigens of GA2, GA5 and GB3, using the Luminex Multiplex system, in volunteers with respiratory disease separated into infected and uninfected groups solely by virus isolation. A similar level of subgroup specific and genotype specific antibodies was found in both infected and uninfected groups. In this study the distribution of cases and ages of infants across the epidemic was not taken into consideration.

The presence of comparable levels of antibodies to GA2G and BAG regardless of the infecting genotype was probably due to the prevalence of genotypes GA2 and BA4 over many epidemics. Epidemiological studies in Newcastle upon Tyne have shown that the two HRSV genotypes tested have been circulating for at least 3 epidemics and probably many epidemics before that (refer to section 10.1.2) and these genotypes were also found in other parts of the world during the similar period. Thus, mothers would have been constantly exposed to these two genotypes in each epidemic and might be expected to have raised sufficient anti-G antibodies against all three genotypes which may explain the apparently good correlation of antibody ODs between GA2G and BAG (section 9.5) and by Vieira et al., (2007). Alternatively, these antibodies could be truely cross-reactive as reinfection by an identical genotype as the initial infection in infants has been reported to mediate production of cross-reactive antibodies at least to the same subgroup (Scott *et al.*, 2007).

It is clear that both anti-F and anti-G antibodies capable of recognising both A and B virus sub-groups and multiple lineages within them and are transferred from mothers to their infants. Most infants are spared from severe HRSV disease in the first month of life corresponding to the higher maternal antibody level. None-the-less most infants admitted to hospital with severe HRSV have detectable serum titers of both anti-F and anti-G antibodies. It appears that there is a threshold antibody level for protection and maternal antibodies must, inevitably drop below this for all infants as maternal anti

bodies decay. When this happens, given the ubiquity of the virus, HRSV infection is almost inevitable. There is evidence that infants hospitalised with HRSV bronchiolitis correlates with low levels of antibodies to the membrane glycoproteins (Ogilvie *et al.*, 1981) although (Kasel *et al.*, 1987) have reported that low anti-F antibody levels also correlate with hospitalisation, in the current study, where antibodies to the G glycoprotein were measured against relevant virus strains, no differences in the ratio of anti-F to anti-G antibodies between index and comparison groups was observed. Thus it appears likely that susceptibility correlates with a general lack of anti-glycoprotein antibodies and the target specificity of the protective antibody species remains un-defined. Whilst the successful deployment of Palivizumab confirms the protective efficacy of anti-F antibodies, more work is justified to understand the function of anti-G antibodies *in vivo* in which protection by anti-G antibodies might be governed by mechanisms yet to be understood.

Chapter 11 Conclusion

Human respiratory syncytial virus infection induces incomplete immunity and results in repeated infections throughout life without many developing severe lower respiratory tract infection. However, a small percentage of HRSV infected infants, progressed on from upper respiratory tract infection to acute lower respiratory tract infection ranging from bronchiolitis to pneumonia. Studies have shown that infants with high level of HRSV specific maternal antibodies tend to be spared from severe lower respiratory tract infection. High titered antibodies are made against two major glycoprotein namely the fusion (F) and G glycoproteins. Nevertheless, the use of anti-fusion glycoprotein prophylaxis only provides average benefit. Thus the question arises whether anti-G antibodies or more specifically anti-G antibodies against the infecting genotype is protective. Thus, it was necessary to develop a system for measuring antibodies against the infecting HRSV genotype in infants admitted to hospital with HRSV infection and a comparison group of similar age but without HRSV infection. The purpose of this study was to assess the efficacy of anti-G antibodies in conferring protection to infants against severe HRSV diseases.

The first aim (Aim I) of this thesis which was to establish the molecular epidemiology of HRSV in Newcastle throughout the three epidemics was straightforward. Two primers sets were used to amplify the C-terminal sequences of the RSV G glycoprotein gene; GC1-F164 for subgroup A and GB1-GB2 for subgroup B. However, GB1-GB2 primer set were found to amplify subgroup A HRSV in nested PCR. As this positive amplification is not specific to subgroup B HRSV. Amplicands were sequenced and genotypes were identified by phylogenetic analysis of the resulting sequences. Over the span of three epidemics, these studies revealed the circulation of three genotypes namely GA2, GA5 and BA4. GA5 was shown to fade into extinction while GA2 was the predominant strain over the first two epidemics, succeeded by BA4 during the third epidemic.

The G glycoprotein genes of the most prevalent genotypes of HRSV, GA2 and BA4, and the F glycoprotein gene of genotype GA2 were isolated and cloned for expression in MVA. While preparing recombinant fusion glycoprotein in MVA under the control of the vaccinia virus p7.5 promoter in HeLa, a rapid formation of giant cells was observed. This phenomenon was not observed while growing the recombinant MVA expressing the F glycoprotein in QT35. This rapid formation of syncytia has not been reported elsewhere. In this study, the mechanism of the formation syncytia was not further explored. Nevertheless, immunofluorescent assay from the time of inoculation

has shown a relatively rapid synthesis of the recombinant F glycoprotein suggesting this phenomenon was caused by newly synthesized recombinant F but the effect of high titered inoculums could not be completely ruled out. Both recombinant G and F glycoproteins were found to bind to ConA and ConA capture ELISA was successfully devised for the measurement of antibodies to both glycoproteins. Thus, Aim II was fulfilled.

As for the third aim (Aim III), an attempt to design a serologic tool for the measurement of antibodies specific to the conserved epitopes of the G glycoprotein was halted as there were overlaps in some conserved and strain specific epitopes. This discovery indicates the difficulty or even impossibilities of devising comprehensive and unique measurements of antibodies to conserved epitopes by direct means via the peptides or recombinant fragments of the G glycoprotein as antigen.

As for the fulfillement of the fourth aim (Aim IV), the measurement of maternal antibodies to the G glycoprotein showed that hospitalized infants were not deficient in HRSV-specific maternal antibodies relative to the comparison group but instead showing a significantly higher antibody level to the G and F glycoproteins of the contemporary strains in the index group compared to the controls. The results indicated that the infants with severe HRSV disease were not specifically deficient in anti-G maternal antibodies.

These findings rejected the hypothesis of this study as a significantly higher level of maternal antibodies to the genotype specific G glycoprotein does not correlate with protection in infants. In addition to this, a significantly higher level of anti-F maternal antibodies were also detected in the index infants which contradicted to the widely accepted idea that anti-F antibodies correlated with immunity. However, a more subtle correlation of anti-F or even anti-G antibody may emerge if a larger sample size was used. Despite the small sample size, the significantly higher antibody level in the index group relative to the comparison group suggests that these findings that reject the hypothesis is unlikely to be false.

Chapter 12 Appendix

12.1 **List of Suppliers**

Applied Biosystems, Carlsbad, California, USA

Astell Scientific Ltd, Kent, DA14 5DT, UK

BDH, VWR International, Leicestershire, UK

Bioline, London, UK

Bio-Rad Laboratories Ltd. Hertfordshire, UK

Cargille Laboratories, Cedar Grove, NJ, USA

Coleman Technologies Inc., Wilmington, USA

Corning, New York, USA

Dade-Behring, Illinois, USA.

Dako, Cambridgeshire, UK

Decon Laboratories Ltd, East Sussex, UK

DuPont (U.K.) Ltd, Herts, UK

Eurofins MWG Operon, http://www.eurofinsdna.com/

Fermentas, York, UK

Fisher Scientific, Leicestershire, UK

Fisons, Leicestershire., UK

Fluka, http://www.sigmaaldrich.com/

GATC Biotech, Lake Constance, Germany

Genevision Ltd, Newcastle upon Tyne, UK

Gibco, Paisley, UK

Hendley Ltd, Essex, UK

Lonza, Rockland, ME, USA

Macherey-Nagel, Düren, Germany

Medichem Int, Kent, UK

Melford Laboratories Ltd, Suffolk, UK

Millipore, Billerica, MA

MJ Research Inc. Watham, MA, USA

Nalgene, Roskilde, Denmark

National Diagnostics, Atlanta, Georgia, USA

Nikon, www. Europe-nikon.com/en_GB/

Northumbria Biologicals Ltd, Cramlington, UK

Novocastra Laboratories, Newcastle upon Tyne, UK

OXOID, Basingstoke, Hampshire, England

PAA, Pasching, Austria

Sigmaplot, www.sigmaplot.com

SPSS Statistics, http://www.spss.com/uk/

Sigma-Aldrich, http://www.sigmaaldrich.com/united-kingdom.html

Starlab Group, Milton Keynes, UK

Swann-Morton® Ltd, Sheffield, England

Weiss-Gallenkamp, Leicestershire, UK

12.2 **Materials**

12.2.1 **Sterilization**

Virkon

1 tablet of Rely+On™ Virkon Tablet (Cat#D12557249) (DuPont) was dissolved in 500 ml of distilled water.

Trigene Advance

Trigene Advance concentrate (Cat#TM305) (Medichem Int.) was diluted 1/100 in distilled water.

12.2.2 **Cell culture**

Phosphate Buffered Saline (PBS)

1 tablet of phosphate buffered saline (Dulbecco A) (OXOID) was dissolved in 100 ml of water, sterilised and stored at ambient temperature.

Heat inactivated Foetal Calf Serum (HI-FCS)

Foetal Bovine Serum Standard Quality (Cat#A15-101) (PAA) was heat inactivated in a water bath at 56 °C for 30 minutes and stored as aliquots of 50 ml at -20 °C.

Trypsin

2.5 % trypsin was supplied as a stock and stored frozen at -20 °C. Stock trypsin was diluted 1/100 in versene and stored at 4 °C until use.

L-glutamine

L-glutamine (Cat# M11-004), (PAA) was purchased as a 200 mM stock and stored frozen at -20 °C. The stock solution was diluted 1/100 in media immediately before use.

Penicillin/Streptomycin Solution

PEN-STREP (Cat#DE17-603E) (LONZA) was purchased as a stock containing 5000 U/ml of Penicillin and 5000 U/ml of Streptomycin and stored frozen at -20 °C. The stock was diluted 1/100 in media immediately before use.

Versene

25g of Ethylenediaminetetraacetic acid (EDTA) was dissolved in 1 litre of PBS, aliquoted in 100 ml volumes, autoclaved and stored at 4°C until use.

Tryptose Phosphate Broth (TPB)

29.5g of TPB (Cat# CM0283) (OXOID) was dissolved in 1 litre of distilled water, aliquoted in 20 ml volumes, autoclaved and kept at ambient temperature.

Non-essential amino acids (NEAA)

Stock NEAA (Cat#M11-003) (PAA) was stored frozen at -20 °C. Stock was thawed to 4 °C and diluted 1/100 in media before use.

0.4 % (w/v) Phenol Red

0.8 g of phenolsulfonphthalein sodium salt (Cat#P5530) (Sigma) was dissolved in PBS and stored at 4 °C until use.

Gassed sodium bicarbonate buffer

44 g of $NaHCO_3$ (Cat#S/4240/60), Fisher Scientific and 2.5 ml of 0.4 % phenol red solution was dissolved in 1 litre of water and held on solid carbon dioxide. The solution was gassed by bubbling 5 % CO_2 through it until a peach coloured solution was produced. It was then aliquoted in 5 ml volumes in glass bijou bottle, autoclaved and stored at ambient temperature.

Tissue culture flasks

All tissue culture flasks were purchased from Corning. T25, T75 and T225 were routinely used and the volume of medium used was 5 ml, 10 ml and 25 ml respectively.

EMEM

Eagle's Minimum Essential Medium (EMEM) (Cat#BE12-684F) (LONZA) was purchased as a 10X stock solution without L-glutamine and $NaHCO_3$ and stored at 4 °C. Stock solution was diluted 1/10 in sterile milli-Q water before use.

GMEM

Glasgow Minimum Essential Medium (GMEM) (Cat#21710-025) (GIBCO) was purchased as a 1X ready to use solution with L-glutamine but without $NaHCO_3$ and stored at 4 °C.

OPTIMEM

OPTI-MEM®I+GlutaMAX™ (OPTIMEM) (Cat#51985-026) (GIBCO) was purchased as a 1X ready to use solution and stored at 4 °C.

HBSS

Hanks Balanced Salt Solution (HBSS) (LONZA) was purchased as 500 ml ready to use 1X solution with Phenol Red, without Calcium and Magnesium and stored at 4 °C until use.

Embryo washing buffer

90 ml of sterile water

10 ml of 10X EMEM

5 ml of Gassed 4.4% Sodium bicarbonate +0.4% phenol red

Growth Medium (GM1)

450 ml of sterile water

50 ml of 10X EMEM

5 ml of NEAA

5 ml of L-glutamine

20 ml of Gassed 4.4% Sodium bicarbonate +0.4 % phenol red

50 ml of HI-FCS

50 ml of TPB

Maintenance medium (MM1)

450 ml of sterile water

50 ml of 10X EMEM

5 ml of NEAA

5 ml of L-glutamine

20 ml of Gassed 4.4% Sodium bicarbonate +0.4 % phenol red

10 ml of HI-FCS

50 ml of TPB

Digestion buffer

Trypsin 0.25 % (v/v) in versene

Cryo buffer

20 ml of FCS (non heat inactivated)

10 ml of DMSO

70 ml of 1X EMEM

Growth medium (GM2)

Formulated as for GM1 (see above) without TPB and NEAA

Maintenance medium (MM2)

Formulated as for MM1 (see above) without TPB and NEAA

Growth medium (GM3)

10 ml of HI-FCS

90 ml of 1X GMEM

10 ml of TPB

1 ml of Penicillin/streptomycin (100 µg/ml)

Maintenance medium (MM3)

2 ml of HI-FCS

90 ml of 1X GMEM

10 ml of TPB

1 ml of Penicillin/streptomycin (100µg/ml)

12.2.3 **Immunofluorescent**

10X Phosphate buffered saline (PBS)

160 g of Sodium chloride (Cat#S/3160/65), Fisher

4 g of Potassium Dihydrogen orthophosphate (Cat#P/4800/60), Fisher

23 g of Disodium hydrogen orthophosphate (Cat#S/45120/60), Fisher

4 g Potassium chloride (Cat#101983K), BDH

Mixed and dissolved in 1.5 litres of water and the pH was adjusted to 7.4 with HCl and volume adjusted to 2 litres at with distilled water. Stock PBS was sterilized and stored at ambient temperature. Dilute 1/10 in distilled water before use.

Evans blue counterstain

Evan's blue counterstain (0.5 % w/v) (Cat#E0133) was diluted 1/1000 in PBS, vortex mixed and stored at 4 °C until use.

10X carbonate coating buffer

15.898 g of Sodium carbonate (Cat#10240), BDH

29.4 g of Sodium bicarbonate (Cat#30151), BDH

Dissolved in 600 ml of distilled water and pH adjusted to 9.6 with 6N NaOH, autoclaved and stored at 4 °C. Diluted 1/10 in distilled water, readjust pH to 9.6 and use immediately.

Serum and antibiotic free medium (SAF)

Formulated as GM2 but without HI-FCS and antibiotics.

12.2.4 **ELISA**

Concanavalin A

25 mg of lyophilized Concanavalin A (ConA) (cat#C2010-25MG) was dissolved in 10 ml of PBS to make 2.5 mg/ml stock solution and stored as 500 µl aliquots at -20 °C until use.

Wash buffer with 0.05% (v/v) Tween20 (PBST)

500 µl of Tween®20 (Cat#P1379-500) dissolved in 1 L of PBS.

Wash buffer with 0.1% Triton X-100 (PBSTx)

1 ml of Triton X-100 (Cat#X-100) dissolved in 1L of PBS.

Blocking buffer (PTF)

10 ml of HI-FCS

90 ml of sterile PBST

Mixed and stored as 20 ml aliquots in glass universal bottles at -20 °C and thawed to 37 °C before use.

Substrate buffer

5.1 g of citric acid and 9.16 g of anhydrous Na2HPO4 were dissolved in 500 ml of distilled water and volume adjusted to 1 litre with distilled water, autoclaved and stored at ambient temperature.

Ortho-phenylendiamine dihydrochloride (OPD) stock solution

1.25 g of OPD was dissolved in 20 ml of substrate buffer to give a final concentration of 62.5 mg/ml. Aliquots of 0.4 ml containing 25 mg of OPD were then stored at -20 °C.

Developer solution

400 µl of OPD was thawed and added to 24.6 ml of substrate buffer followed by 10 µl of 30 % (v/v) hydrogen peroxide (BDH) and used immediately.

3M H_2SO_4

50 ml of concentrated H_2SO_4 1.84 SG (Cat#450063S) (BDH) was gently titrated into 150 ml of ice cold distilled water in an iced water bath.

12.2.5 **SDS-PAGE**

40% Acrylamide Solution

Ready to use 40 % acrylamide solution (Cat#BP1408-1) (Fisher) was stored at 4 °C in the dark until use.

Resolving buffer (1.5M Tris-Cl, pH8.8)

272.3 g Tris base (Cat#B2005), (Melford Lab. Ltd.) was dissolved in 800 ml of water and the pH adjusted to 8.8 with concentrated HCl. The solution was sterilized and stored at ambient temperature until use.

Stacking buffer (0.5M Tris-HCl, pH6.8)

30 g Tris base (Cat#B2005), (Melford Lab. Ltd.) was dissolved in 400 ml of water and the pH was adjusted to 6.8 with concentrated HCl. The solution was sterilized and stored at ambient temperature until use.

10 % (w/v) SDS

10 g of sodium dodecyl sulphate (Cat#L-4509) was dissolved in 80 ml of water and volume was adjusted to 100 ml in a volumetric flask.

The solution was stored at ambient temperature until use.

Blocking buffer

10 g of Skimmed milk powder was dissolved in 100 ml of PBST to make 10 % (w/v) solution and used immediately.

Tris buffered Saline (TBS) pH 7.4

8 g NaCl

0.2 g KCl

0.3 g Tris base

Dissolved in 800 ml of distilled water, pH adjusted to 7.4 with HCl, sterilized and stored at room temperature until use.

4X reducing sample buffer

3.8 ml of distilled water

1 ml of 0.5M Tris-HCl, pH6.8

0.8 ml of glycerol

1.6 ml of 10% SDS

0.4 ml of 2-mercaptoethanol (Cat#63689) (Fluka)

0.4 ml of 1 % (w/v) bromophenol blue

Combined and stored as 1ml aliquots at -20 °C. Thaw to ambient temperature before use.

1X reducing sample buffer

65 µl of PBS,

10 µl of protease inhibitor

25 µl of 4X reducing sample buffer

Combine and used immediately to treat for one sample.

5X Electrode running buffer

15 g of Tris base

72 g of Glycine, Fisher (Cat#BP381-5)

5 g of SDS

Dissolved in water to make l litre solution and kept at ambient temperature. Diluted 1/5 with distilled water and chilled to 4 °C before use.

10 % (w/v) Ammonium Persulphate (APS)

100 mg of APS (Cat#A/P470/46) (Fisher) was dissolved in 1 ml of water and stored at 4 °C for a maximum of 2 weeks before use.

N,N,N',N'-tetramethylethylenediamine (TEMED)

Ready to use TEMED (Cat#EC503) (National Diagnostics)

Substrate A

0.15 g of 4-chloro-1-napthol (4CN) (Cat#C8890)

150 ml of Methanol (Cat#M/4000/17), Fisher

50 ml of TBS pH 7.4

Dissolved and stored as aliquots of 10ml in universal bottles at -20 °C. Thawed to room temperature before use.

Substrate B

10 µl of 30% Hydrogen peroxide, BDH (Cat#101284N)

10 ml of TBS pH7.4

Substrate A and B

Equal volumes of substrate A and substrate B were combined and used immediately.

10 % Resolving gel

2.5 ml of 40 % acrylamide

2.5 ml of 1.5 M Tris pH 8.8

0.1 ml of 10 % Ammonium persulphate

0.1 ml of 10 % SDS

0.004 ml of TEMED

4.8 ml of water

5 % Stacking gel

0.625 ml of 40 % acrylamide

0.625 ml of 1.5 M Tris pH 8.8

0.05 ml of 10 % Ammonium persulphate

0.05 ml of 10 % SDS

0.005 ml of TEMED

3.65 ml of Water

12.2.6 **Western blotting**

10X Towbin buffer

30.3 g Tris base

144 g Glycine

Dissolved in 1 liter of distilled water, sterilized and stored at ambient temperature.

1X Towbin buffer

100 ml of 10X Towbin buffer

200 ml of Methanol

Volume adjusted to 1 liter with distilled water and stored at 4 °C until use.

12.2.7 **Modified Lowry Assay**

Reagent A

2 g of Na_2CO_3 (Cat#10240) (BDH)

0.4 g of NaOH (pearl) (Cat#104384F) (BDH)

0.16 g of Sodium Tartrate dibasic dehydrate (Cat#4797-100G)

1 g of SDS

Dissolved in 80 ml of water and volume adjusted to 100 ml.

Stored at ambient temperature until use.

Reagent B

Dissolved 4 g of CuSO4.5H2O (Cat#C7631-250G) was dissolved in 100 ml of water and stored at ambient temperature.

Reagent C

100 parts of Reagent A + 1 part of Reagent B prepared fresh before use.

Reagent D

1 part of 2 N Folin Ciocalteu's phenol reagent (Cat#F9252-500 ml) + 1 part of water. Prepared fresh before use.

Lysis Buffer

0.5% (v/v) Triton X-100 was dissolved in PBS, autoclaved and stored at ambient temperature.

Protein standard

500 mg of bovine serum albumin Cohn Fraction V, pH7.0 (Cat#422371X) (BDH) was dissolved in 50 ml of Lysis buffer as described above to make 10mg/ml stock and stored as 5 ml aliquot in plastic bijou bottle at -20 °C until use.

Dilutions of 10, 5, 2.5, 1, 0.5, 0.3, 0.2, 0.1, 0.05 and 0 mg/ml were prepared as standards in 1 ml volumes with lysis buffer and used immediately.

12.2.8 **Biorad Protein Assay**

Protein Standard

Protein standards were prepared as described in section 3.13 but was dissolved in PBS supplemented with 0.1 % (w/v) SDS.

Biorad Assay reagent

Biorad Protein Assay Dye reagent concentrate (Cat#500-0006) concentrate is stored at 4 °C.

1.75 ml of Biorad Reagent and 5.25 ml of water were mixed and used immediately.

12.2.9 **Molecular Biology**

Diethyl pyrocarbonate treated water (DEPC-H_20)

All distilled water used in the molecular methodologies was treated with 0.1 % (v/v) diethyl pyrocarbonate (DEPC), BDH (Cat#44170 3D) overnight at 37 °C water bath, sterilized and stored at ambient temperature until use.

10X Tris, Boric acid, EDTA (TBE) solution

108 g of Tris base

55 g of Boric acid (Cat#B6768)

9.3 g of EDTA

Autoclaved and stored at room temperature. Dilute 1/10 in distilled water before use.

6X Loading dye

30 ml Glycerol

0.1 g bromophenol blue

0.1 g xylene cyanole FF

Dissolved in 100 ml of DEPC-H_20 and stored as 1 ml aliquots at -20 °C and thawed to ambient temperature before use.

dNTPs

dNTPs stock was prepared from dNTP set (Cat#R0181) (Fermentas) by combining 250 µl of each dNTPs together and mixed with 1500 µl of DEPC-H_2O to make 10 mM stock solution and stored as aliquot of 200 µl at -20 °C until use.

Ethidium bromide (EtBr)

10 mg/ml (w/v) EtBr stock solution (E/P800/03) (Fisher) was stored at 4 °C and diluted in TBE buffer to 10 µg/ml working concentration immediately before use.

12.2.10 **Bacteriology**

All chemicals in this section were purchased from OXOID unless stated otherwise.

All restriction enzymes were purchased from Fermentas.

T4 DNA ligase was purchased from Invitrogen.

Luria-Bertani broth (LB)

10 g of Tryptone (Cat#LP0021)

5 g of Yeast extract (Cat#LP0042)

10 g of Sodium chloride (Fisher Scientific) (Cat#2315983)

The volume was adjusted with distilled water to 1 litre, autoclaved and stored at ambient temperature.

Luria-Bertani Agar (LBA)

4 g of Tryptone

2 g of Yeast extract

4 g of Sodium chloride

6 g of Agar Technical (Agar No.3) (Cat#LP0013)

The volume was adjusted to 400 ml with distilled water in a 500 ml Duran bottle, sterilized and stored at ambient temperature.

LBA was melted in a boiling waterbath, equilibrated to 60 °C in a waterbath set at that temperature. Ampicillin was added to the final concentration of 100 µg/ml where necessary at this stage before pouring. Agar plates were prepared by pouring directly on the petri dish until the molten agar covers the whole surface (Approximately 15-20 ml/dish). Agar plates were cooled to ambient temperature in a biological safety cabinet for 1 hour before storeing in sealed plastic bags at 4 °C until use.

SOB medium

20 g of tryptone

5 g of yeast extract

0.5 g of NaCl

0.186 g of KCl

The volume was adjusted to 1 litre, autoclaved and stored at 4°C.

1 M $MgCl_2$

9.521 g of anhydrous $MgCl_2$ (Cat#M8266) was dissolved in 50 ml of distilled water and the volume adjusted to 100ml in a volumetric flask, autoclaved and stored at 4 °C.

1M $MgSO_4$

24.647 g of $MgSO_4.7H_2O$ (Cat#BP213.1) (Fisher) was dissolved in 50 ml of distilled water and the volume adjusted to 100 ml in a volumetric flask, autoclave sterilized and stored at 4 °C.

1 M glucose

9.08 g of D-glucose (Cat#1011744) (BDH) was dissolved in 30 ml of distilled water and the volume adjusted to 50 ml, filter sterilized and stored at -20 °C in a 50 ml centrifuge tube until use.

SOC medium

100 ml of SOB was supplemented with 100 µl of 1 M $MgCl_2$, 100 µl of 1 M $MgSO_4$ and 200 µl of 1 M D-glucose. The solution was stored at -20 °C in aliquots of 20 ml in disposable plastic universal bottles.

Ampicillin

1 g of Ampicillin sodium salt (Cat#A9518-5G) was dissolved in 10 ml of water to make 100 mg/ml stock solution. The solution was filter sterilized and stored as 1 ml aliquot in 2 ml vial at -20 °C until use.

Tfb1 solution

0.296 g of CH_3COOK (Cat#P1190)

1.210 g of RbCl (Cat#R2252)

0.148 g of $CaCl_2.2H_2O$ (Cat#C3881)

1.000 g of $MnCl_2.4H_2O$ (Cat#13446-34-9)

Dissolved in 50 ml of water and pH adjusted to 5.8 using acetic acid.

15 ml of glycerol was added and volume adjusted to 100 ml with water in a volumetric flask. The solution was filter sterilized and stored at -20 °C until use.

Tfb2 solution

0.105 g of MOPS (Cat#M3183)

0.060 g of RbCl (Cat#R2252)

0.550 g of $CaCl_2.2H_2O$ (Cat#C3881)

Dissolved in 20 ml of water and the pH adjusted to 6.5 with KOH.

7.5 ml of glycerol was added, mixed and the volume adjusted to 50 ml in a volumetric flask.

12.2.11 Cell titration and plaquing

10% formal saline

9 g of sodium chloride was dissolved in 800 ml of distilled water and 100 ml of formaldehyde was added. The volume was adjusted to 1 litre with distilled water and the solution was stored at ambient temperature.

Crystal violet solution

0.1 g of crystal violet was dissolved in 80 ml of distilled water and the pH was adjusted to 7.0 with ammonia. The solution was stored at ambient temperature.

Neutral red agarose

Neutral red (Cat# N4638) was diluted 1:3 in PBS to make 1.5 ml solution and combined with 13 ml of molten 2 % Agarose Type VII.

2% (w/v) Agarose type VII

2 g of Agarose type VII (Cat#A9045) was added to 80 ml of distilled water in 100 ml Duran bottle, autoclave sterilized and stored at ambient temperature.

For use, agarose was melted in a boiling waterbath and held in a 37 °C incubator in its molten state until use.

2X EMEM

20 ml of 10X EMEM

20 ml of TPB

10 ml of gassed sodium bicarbonate buffer

2 ml of 100X Penicillin/streptomycin

4 ml of HI-FCS

42 ml of water

The solution was stored at 4 °C up to 3 months. For use, the desired volume was aliquoted into a plastic universal bottle and L-glutamine was added to the final concentration of 2 µg/ml (2X concentration). The solution was then warmed to 37 °C before use.

Xgal

1 mg/vial of Xgal, Fermentas (Cat#R404) was dissolved in 10ml of dimethylformamide (DMF) (Cat#D4551) to make 100mg/ml stock. The solution was stored at -20 °C in 2 ml aliquots in glass bijou bottle until use.

Agarose overlay

Combine equal volume of 2 % (w/v) Agarose type VII and 2 X EMEM as described above, mixed by gentle inversion immediately before use. The minimum agarose overlay required for 24 wells plate and 6 wells plate is 500 µl and 1000 µl per well respectively.

Xgal Agarose overlay

Xgal stock was diluted to 400 µg/ml in 2X EMEM and formulate as described in Agarose overlay (see above).

12.3 **Isolates**

Table 24 Summary of the isolation and subgrouping of HRSV isolated during the 2007/2008 epidemic in Newcastle upon Tyne

No.	Isolate NPS	Anti-RSV Mabpool	Subgroup
1	40/08	+	A
2	41/08	+	A
3	42/08	+	A
4	44/08	+	A
5	45/08	+	B
6	46/08	+	A
7	48/08	+	A
8	49/08	+	A
9	50/08	+	A
10	51/08	+	A
11	53/08	+	A
12	54/08	+	B
13	55/08	+	A
14	56/08	+	B
15	57/08	+	A
16	58/08	+	A
17	59/08	+	A

Table 25 Summary of the isolation and subgrouping of HRSV isolated during the 2008/2009 epidemic in Newcastle upon Tyne

	Isolate	IF	Genotyping
1	0309	+	BA4
2	0409	+	GA5
3	0609	+	BA4
4	0709	+	GA2
5	0909	+	GA2
6	1109	+	BA4
7	1209	-	Not RSV
8	1309	N/A	Isolation negative
9	1409	+	GA2
10	1509	N/A	Isolation negative
11	1709	N/A	Isolation negative
12	1809	N/A	Isolation negative
13	1909	N/A	Isolation negative
14	2009	N/A	Isolation negative
15	2109	+	BA4
16	2209	N/A	Isolation negative
17	2409	+	GA2
18	2509	+	BA4
19	2709	N/A	Isolation negative
20	2809	+	GA2
21	2909	+	GA2
22	3009	+	unknown
23	3109	+	GA2
24	3209	N/A	Isolation negative
25	3309	N/A	Isolation negative
26	3409	N/A	Isolation negative
27	3609	-	Not RSV
28	3909	+	GA2
29	4009	+	GA2

N/A- not attempted

Table 26 Summary of specimens obtained from infant volunteers over epidemic 2009/2010

#	Study No.	Date of Birth	Date of collection	Age	Sample type	Approx serum volume (µl)	Isolation/ passage	RT-PCR/ sequencing	IF with anti-RSV Mabpool
1	16	17/10/2009	02/12/2009	46	serum	50ul			
			02/12/2009		NS	200ul	P1	GA2	POS
			03/12/2009		NPS	200ul	P1	GA2	POS
2	27	24/10/2009	06/12/2009	43	serum	60ul			
			06/12/2009		NS	500ul	P1	GA2	POS
3	41	14/10/2009	23/12/2009	70	serum	30ul			
		14/10/2009			NPS	150ul	P1	GA2	POS
4	51	18/11/2009	11/01/2010	54	serum	50ul			NEG
					NS	500ul	NEG	GA2φ	
5	30	24/10/2009	07/12/2009	44	NPS	200ul	P1	BA	POS
		24/10/2009	07/12/2009	44	serum	50ul			
		24/10/2009	09/12/2009	46	serum				
6	35	15/10/2009	16/12/2009	62	serum	60ul			
					NPS	150ul	P1	BA	POS
7	44	08/10/2009	28/12/2009	81	serum	50ul			
					NS	500ul	P1/P2	BA	POS
8	45	19/08/2009	30/12/2009	133	serum	70ul			
					NS	500ul	P1	BA	POS
9	47	05/07/2009	03/01/2010	182	serum				
					NS	500ul	P1/P2	BA	POS
					NPS	~1ml	P1	BA	POS
10	49	19/07/2009	11/01/2010	176	Blood				
					NS		P1	BA	POS
11	53	12/09/2009	20/01/2010	130	NPS		P1	BA	POS
					Blood	120ul			
12	52	13/12/2009	18/01/2010	36	blood	50ul			
					NS		NEG	BAφ	NEG
13	54	10/12/2009	18/01/2010	39	blood	20ul	NEG		NEG
					NS			BAφ	
14	29	29/10/2009	07/12/2009	39	NS		NEG	BAφ	NEG
			09/12/2009		serum	50ul			
15	32	11/07/2009	12/12/2009	154	NS	500ul	NEG	BAφ	NEG
					serum	40ul			
16	46	20/10/2009	30/12/2009	71	serum	50ul	NEG	BAφ	NEG

					NS	500ul			
17	50	18/11/2009	17/01/2010	60	NS blood		NEG	BAφ	NEG
18	21	15/01/2010	07/02/2010	23 0	serum NS	50ul 500ul	NEG	BAφ	
19	40	09/08/2009	23/12/2009	136	serum NS	60ul 500ul	NEG	NEG	NEG
20	33	21/10/2009	14/12/2009	54	NS serum	500ul 200ul	NEG	NEGφ	NEG
21	28	19/10/2009	06/12/2009	48	NS serum	50ul	NEG	NEGφ	NEG
22	43	13/11/2009	27/12/2009	44	serum NS	15ul 500ul	NEG	NEGφ	NEG
23	48	10/09/2009	03/01/2010	115	serum NS		NEG	NEGφ	NEG
24	31	11/07/2009	09/12/2009	151	NS	500ul	NEG	NEG	No serum
25	36	02/12/2009	19/12/2009	17	serum	60ul	N/A	N/A	No NPS/NS
26	37	07/12/2009	19/12/2009	12	serum	30ul	N/A	N/A	No NPS/NS
27	38	09/08/2009	22/12/2009	135	serum	120ul	N/A	N/A	No NPS/NS
28	39	21/10/2009	22/12/2009	62	serum	70ul	N/A	N/A	No NPS/NS
29	15	03/08/2009	27/12/2009	146	serum	150ul	N/A	N/A	No NPS/NS
30	19	22/07/2009	14/12/2009	145	NS	500ul	NEG	NEG	No serum
31	34	10/11/2009	13/12/2009	33	serum	70ul	N/A	N/A	No NPS/NS

φ by nested PCR

Table 27 Passage history of isolates.

No.	Isolate NPS	Genotype	Date of collection	P1	P2	comment
1	40/08	GA2	N/A	16-21/1	11-18/2	2007/2008
2	41/08	GA2	N/A	16-24/1	24-28/1	2007/2008
3	42/08	GA2	N/A	16-24/1	13-15/2	Mix virus present
4	44/08	GA2	N/A	28-4/2	8-18/2	2007/2008
5	45/08	GA2	N/A	7-14/2	7-14/2	2007/2008
6	46/08	GA2	N/A	7-14/2	7-14/2	2007/2008
7	48/08	GA5	N/A	22-28/2	29-3/3	2007/2008
8	49/08	GA5	N/A	28-10/3	11-18/3	2007/2008
9	50/08	GA2	N/A	28-17/3	20-25/3	2007/2008
10	51/08	GA2	N/A	28-17/3	20-25/3	2007/2008
11	53/08	GA2	N/A	11-19/3	20-25/3	2007/2008
12	54/08	GA2	N/A	20-3/4	4-10/4	2007/2008
13	55/08	GA2	N/A	20-3/4	4-10/4	2007/2008
14	56/08	GA2	N/A	17-24/4	24-28/4	2007/2008
15	57/08	GA2	N/A	8-15/5	15-19/5	2007/2008
16	58/08	GA2	N/A	13-23/5	23-30/5	2007/2008
17	59/08	GA2	N/A	20-27/5	27-30/5	2007/2008
18	03/09	BA4	17/11/08	4-11/2	N/A	2008/2009
19	04/09	GA5	17/11/08	4-11/2	22-2/2	2008/2009
20	06/09	BA4	19/11/08	N/A	8-14/4	2008/2009
21	07/09	GA2	19/11/08	28-30/1	N/A	2008/2009
22	09/09	GA2	28/11/08	28-2/2	4-11/2	2008/2009
23	11/09	BA4	28/11/08	28-2/2	4-11/2	2008/2009
24	14/09	GA2	4/2/09	4-16/2	4/3-8/3	2008/2009

25	21/09	BA4	11/2/09	11-24/2	8-17/4	2008/2009
26	24/09	GA2	18/2/09	18-26/2	1/3-8/3	2008/2009
27	25/09	BA4	18/2/09	18-4/3	N/A	2008/2009
28	28/09	GA2	4/3/09	4-9/3	N/A	2008/2009
29	29/09	GA2	4/3/09	4-10/3	N/A	2008/2009
30	30/09	BA4	4/3/09	4-10/3	N/A	2008/2009
31	31/09	GA2	4/3/09	4-10/3	N/A	2008/2009
32	39/09	GA2	7/4/09	8-17/4	N/A	2008/2009
33	40/09	GA2	15/4/09	15-19/4	N/A	2008/2009
34	16/10	GA2	2/12/09	2-7/12	N/A	2009/2010
35	27/10	GA2	06/12/09	14-28/12	N/A	2009/2010
36	41/10	GA2	23/12/09	23-5/1	N/A	2009/2010
37	30/10	GA2	07/12/09	7-10/12	N/A	2009/2010
38	35/10	BA4	16/12/09	16-24/12	N/A	2009/2010
39	44/10	BA4	28/12/09	28-12/1	N/A	2009/2010
40	45/10	BA4	30/12/09	30-12/1	N/A	2009/2010
41	47/10	BA4	03/01/10	4-21/1	N/A	2009/2010
42	49/10	BA4	11/01/10	12-20/1	N/A	2009/2010
43	53/10	BA4	20/01/10	20-4/2	N/A	2009/2010

Reading the Isolate name: Isolate 40/08 means isolate from NPS #40 isolated in year 2008 in season 2007/2008.

Dates stated for isolates season 2007/2008-2008-2009 corresponds to the day of inoculation till the day of harvest.

Dates stated for isolates from season 2009/2010 corresponds to the day where the specimen was taken.

12.4 Sequences used in the construction of the subgroup A phylogenetic tree

>NCL/51/10
AAGAAAACCACCACCAAGCCCACAAAAAAACCAACCATCAAGACAACCAAAAAAGATCCC
CAACCTCAAACCACAAAACCAAAGGAAGTACTCACCACCAAGCCCACAGAAAAGCCAACCA
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AGAACACACAAGTCAAGAGGAAACCCTCCACTCAACCACCTCCGAAGGCAATCTAAGCCCA
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12.5 Sequences used in the construction of the subgroup B/BA phylogenetic tree

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12.6 **Complete G and F in pSC11**

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12.7 **Truncation**

Engineered restriction sites, NcoI and BamHI are boxed.

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12.8 **ELISAs**

Table 28 Direct IgA ELISA with infant sera.

	A2			HeLa			CORRECTED				
ID	Read1	Read2	Read3	Read1	Read2	Read3	Read1	Read2	Read3	Mean	SD
21	1.767	1.788	1.633	0.333	0.353	0.437	1.434	1.435	1.196	1.355	0.1377
58	0.242	0.285	0.247	0.255	0.264	0.290	-0.013	0.021	-0.043	-0.012	0.03202
55	0.282	0.284	0.304	0.260	0.258	0.302	0.022	0.026	0.002	0.017	0.01286
56	0.408	0.315	0.332	0.284	0.314	0.276	0.124	0.001	0.056	0.060	0.06161
65	0.300	0.287	0.251	0.316	0.306	0.305	-0.016	-0.019	-0.054	-0.030	0.02113
20	0.310	0.287	0.288	0.239	0.225	0.284	0.071	0.062	0.004	0.046	0.03636
16	0.277	0.237	0.233	0.281	0.283	0.281	-0.004	-0.046	-0.048	-0.033	0.02485
27	0.525	0.452	0.441	0.365	0.341	0.339	0.160	0.111	0.102	0.124	0.03121
51	0.275	0.218	0.218	0.221	0.226	0.235	0.054	-0.008	-0.017	0.010	0.03866
30	0.238	0.246	0.245	0.279	0.274	0.290	-0.041	-0.028	-0.045	-0.038	0.00889
52	1.853	1.945	1.840	0.345	0.345	0.418	1.508	1.600	1.422	1.510	0.08902
54	0.487	0.496	0.511	0.491	0.525	0.526	-0.004	-0.029	-0.015	-0.016	0.01253
29	0.274	0.274	0.283	0.280	0.280	0.303	-0.006	-0.006	-0.020	-0.011	0.00808
50	0.235	0.236	0.250	0.280	0.267	0.336	-0.045	-0.031	-0.086	-0.054	0.02858
33	0.763	0.741	0.692	0.296	0.311	0.316	0.467	0.430	0.376	0.424	0.04576
28	0.366	0.258	0.246	0.248	0.255	0.242	0.118	0.003	0.004	0.042	0.06611
43	0.648	0.606	0.640	0.284	0.289	0.342	0.364	0.317	0.298	0.326	0.03398
22	0.397	0.397	0.383	0.331	0.337	0.378	0.066	0.060	0.005	0.044	0.03362
60	0.289	0.303	0.308	0.304	0.300	0.344	-0.015	0.003	-0.036	-0.016	0.01952
24	0.343	0.357	0.382	0.322	0.339	0.384	0.021	0.018	-0.002	0.012	0.0125
61	0.612	0.497	0.474	0.330	0.323	0.337	0.282	0.174	0.137	0.198	0.07534
41	0.428	0.334	0.326	0.308	0.325	0.325	0.120	0.009	0.001	0.043	0.06652
35	0.292	0.256	0.254	0.237	0.236	0.243	0.055	0.020	0.011	0.029	0.02325
44	0.391	0.357	0.361	0.422	0.428	0.461	-0.031	-0.071	-0.100	-0.067	0.03465
46	0.339	0.267	0.249	0.252	0.276	0.271	0.087	-0.009	-0.022	0.019	0.05953

18	0.476	0.431	0.415	0.387	0.370	0.354	0.089	0.061	0.061	0.070	0.01617
17	0.450	0.340	0.341	0.315	0.321	0.338	0.135	0.019	0.003	0.052	0.07204
48	0.532	0.554	0.561	0.279	0.277	0.371	0.253	0.277	0.190	0.240	0.04493
2	0.279	0.283	0.287	0.321	0.331	0.395	-0.042	-0.048	-0.108	-0.066	0.0365
25	0.393	0.315	0.303	0.311	0.314	0.314	0.082	0.001	-0.011	0.024	0.05059
26	0.464	0.353	0.313	0.279	0.272	0.260	0.185	0.081	0.053	0.106	0.06955
45	0.370	0.254	0.266	0.292	0.294	0.305	0.078	-0.040	-0.039	0.000	0.06784
53	0.403	0.413	0.409	0.356	0.353	0.432	0.047	0.060	-0.023	0.028	0.04464
40	0.714	0.695	0.653	0.349	0.339	0.334	0.365	0.356	0.319	0.347	0.02438
59	0.274	0.271	0.271	0.301	0.310	0.385	-0.027	-0.039	-0.114	-0.060	0.04715
49	0.389	0.344	0.329	0.296	0.294	0.295	0.093	0.050	0.034	0.059	0.03051
32	0.292	0.306	0.305	0.271	0.280	0.373	0.021	0.026	-0.068	-0.007	0.05289
47	0.323	0.286	0.287	0.310	0.332	0.320	0.013	-0.046	-0.033	-0.022	0.031

Table 29 Maternal antibody against the fusion glycoprotein (GA2F) of HRSV GA2 genotype.

	GA2F			BGAL			CORRECTED				
ID	Read1	Read2	Read3	Read1	Read2	Read3	Read1	Read2	Read3	Mean	SD
21	1.178	1.174	1.078	0.298	0.294	0.487	0.880	0.880	0.591	0.784	0.167
58	2.026	1.976	1.856	0.222	0.222	0.330	1.804	1.754	1.526	1.695	0.148
55	1.521	1.289	1.245	0.341	0.328	0.336	1.180	0.961	0.909	1.017	0.144
56	2.708	2.465	2.330	0.266	0.269	0.255	2.442	2.196	2.075	2.238	0.187
65	2.239	2.243	2.143	0.400	0.407	0.514	1.839	1.836	1.629	1.768	0.120
20	2.821	2.514	2.394	0.157	0.146	0.160	2.664	2.368	2.234	2.422	0.220
16	1.609	1.567	1.486	0.200	0.209	0.306	1.409	1.358	1.180	1.316	0.120
27	1.892	1.643	1.600	0.610	0.530	0.517	1.282	1.113	1.083	1.159	0.107
51	2.676	2.793	2.652	0.233	0.234	0.374	2.443	2.559	2.278	2.427	0.141
30	2.682	2.458	2.383	0.500	0.494	0.484	2.182	1.964	1.899	2.015	0.148
52	1.895	1.509	1.481	0.223	0.217	0.218	1.672	1.292	1.263	1.409	0.228
54	2.462	2.132	1.976	0.240	0.237	0.240	2.222	1.895	1.736	1.951	0.248
29	2.635	2.446	2.331	0.216	0.212	0.198	2.419	2.234	2.133	2.262	0.145
50	0.786	0.554	0.536	0.170	0.166	0.174	0.616	0.388	0.362	0.455	0.140
33	2.487	2.423	2.458	1.644	1.591	1.609	0.843	0.832	0.849	0.841	0.009
28	1.196	1.007	1.000	0.139	0.139	0.143	1.057	0.868	0.857	0.927	0.112
43	1.427	1.361	1.342	0.412	0.385	0.520	1.015	0.976	0.822	0.938	0.102
22	0.744	0.701	0.715	0.171	0.178	0.246	0.573	0.523	0.469	0.522	0.052
60	1.087	1.031	1.009	0.185	0.190	0.325	0.902	0.841	0.684	0.809	0.112
24	2.509	2.472	2.349	0.458	0.468	0.560	2.051	2.004	1.789	1.948	0.140
61	1.566	1.567	1.479	0.566	0.570	0.735	1.000	0.997	0.744	0.914	0.147
41	1.864	1.505	1.484	0.380	0.387	0.391	1.484	1.118	1.093	1.232	0.219
35	1.171	1.188	1.211	0.338	0.287	0.397	0.833	0.901	0.814	0.849	0.046
44	1.156	0.990	0.995	0.422	0.458	0.445	0.734	0.532	0.550	0.605	0.112
46	1.610	1.320	1.354	0.312	0.300	0.282	1.298	1.020	1.072	1.130	0.148

18	0.705	0.610	0.613	0.125	0.118	0.125	0.580	0.492	0.488	0.520	0.052
17	0.485	0.448	0.441	0.319	0.435	0.320	0.166	0.013	0.121	0.100	0.079
48	1.146	0.988	0.962	0.315	0.315	0.317	0.831	0.673	0.645	0.716	0.100
2	0.282	0.285	0.294	0.112	0.102	0.178	0.170	0.183	0.116	0.156	0.036
25	0.519	0.510	0.524	0.575	0.581	0.653	-0.056	-0.071	-0.129	-0.085	0.039
26	0.043	0.045	0.051	0.054	0.056	0.085	-0.011	-0.011	-0.034	-0.019	0.013
45	0.341	0.336	0.322	0.103	0.105	0.197	0.238	0.231	0.125	0.198	0.063
53	2.971	2.735	2.609	0.502	0.474	0.462	2.469	2.261	2.147	2.292	0.163
40	0.280	0.188	0.186	0.168	0.159	0.195	0.112	0.029	-0.009	0.044	0.062
59	1.907	1.755	1.617	0.115	0.120	0.127	1.792	1.635	1.490	1.639	0.151
49	0.478	0.474	0.524	0.383	0.371	0.481	0.095	0.103	0.043	0.080	0.033
32	0.858	0.628	0.630	0.304	0.311	0.304	0.554	0.317	0.326	0.399	0.134
47	1.675	1.511	1.407	0.599	0.602	0.575	1.076	0.909	0.832	0.939	0.125

Table 30 Maternal antibody against the G glycoprotein of HRSV genotype GA2.

	GA2G			BGAL			CORRECTED				
ID	Read1	Read2	Read3	Read1	Read2	Read3	Read1	Read2	Read3	Mean	SD
21	0.546	0.387	0.383	0.348	0.235	0.215	0.198	0.152	0.168	0.173	0.023
58	1.354	1.358	1.352	0.190	0.189	0.296	1.164	1.169	1.056	1.130	0.064
55	0.327	0.286	0.296	0.275	0.273	0.539	0.052	0.013	-0.243	-0.059	0.160
56	2.712	2.686	2.638	0.246	0.247	0.340	2.466	2.439	2.298	2.401	0.090
65	3.222	3.237	3.268	0.349	0.341	0.563	2.873	2.896	2.705	2.825	0.104
20	1.664	1.535	1.458	0.147	0.146	0.144	1.517	1.389	1.314	1.407	0.103
16	1.741	1.545	1.517	0.270	0.247	0.253	1.471	1.298	1.264	1.344	0.111
27	1.352	0.890	0.798	0.359	0.368	0.447	0.993	0.522	0.351	0.622	0.332
51	1.454	1.265	1.299	0.200	0.194	0.193	1.254	1.071	1.106	1.144	0.097
30	1.761	1.641	1.673	0.581	0.548	0.627	1.180	1.093	1.046	1.106	0.068
52	0.659	0.612	0.601	0.174	0.171	0.177	0.485	0.441	0.424	0.450	0.031
54	2.058	2.009	1.924	0.178	0.184	0.184	1.880	1.825	1.740	1.815	0.071
29	2.524	2.483	2.479	0.277	0.280	0.349	2.247	2.203	2.130	2.193	0.059
50	0.965	0.605	0.834	0.201	0.209	0.181	0.764	0.396	0.653	0.604	0.189
33	2.349	2.180	2.132	1.668	1.646	1.581	0.681	0.534	0.551	0.589	0.080
28	0.573	0.564	0.569	0.126	0.124	0.157	0.447	0.440	0.412	0.433	0.019
43	3.092	3.201	2.870	0.275	0.267	0.272	2.817	2.934	2.598	2.783	0.171
22	0.517	0.534	0.516	0.174	0.176	0.238	0.343	0.358	0.278	0.326	0.043
60	0.594	0.590	0.622	0.121	0.124	0.188	0.473	0.466	0.434	0.458	0.021
24	2.348	2.365	2.249	0.193	0.195	0.310	2.155	2.170	1.939	2.088	0.129
61	1.769	1.527	1.517	0.461	0.417	0.378	1.308	1.110	1.139	1.186	0.107
41	1.096	1.105	1.081	0.299	0.289	0.396	0.797	0.816	0.685	0.766	0.071
35	1.219	1.235	1.216	0.195	0.202	0.419	1.024	1.033	0.797	0.951	0.134
44	0.352	0.347	0.345	0.252	0.253	0.445	0.100	0.094	-0.100	0.031	0.114
46	1.150	1.131	1.280	0.212	0.231	0.469	0.938	0.900	0.811	0.883	0.065
18	0.520	0.443	0.442	0.124	0.122	0.122	0.396	0.321	0.320	0.346	0.044

17	0.306	0.210	0.193	0.201	0.193	0.193	0.105	0.017	0.000	0.041	0.056
48	0.797	0.789	0.763	0.231	0.221	0.332	0.566	0.568	0.431	0.522	0.079
2	1.070	0.800	0.827	0.292	0.250	0.290	0.778	0.550	0.537	0.622	0.136
25	0.805	0.588	0.588	0.434	0.427	0.430	0.371	0.161	0.158	0.230	0.122
26	0.110	0.069	0.067	0.064	0.061	0.073	0.046	0.008	-0.006	0.016	0.027
45	0.509	0.327	0.301	0.148	0.141	0.152	0.361	0.186	0.149	0.232	0.113
53	2.943	2.783	2.680	0.228	0.220	0.223	2.715	2.563	2.457	2.578	0.130
40	0.382	0.398	0.370	0.157	0.153	0.274	0.225	0.245	0.096	0.189	0.081
59	0.353	0.352	0.339	0.105	0.108	0.211	0.248	0.244	0.128	0.207	0.068
49	0.422	0.292	0.257	0.178	0.171	0.183	0.244	0.121	0.074	0.146	0.088
32	0.298	0.290	0.285	0.215	0.210	0.283	0.083	0.080	0.002	0.055	0.046
47	0.717	0.538	0.536	0.220	0.215	0.210	0.497	0.323	0.326	0.382	0.100

Table 31 Maternal antibody to the G glycoprotein of HRSV genotype BA4.

	BAG			BGAL			CORRECTED				
ID	Read1	Read2	Read3	Read1	Read2	Read3	Read1	Read2	Read3	Mean	SD
21	0.403	0.404	0.409	0.191	0.188	0.268	0.212	0.216	0.141	0.190	0.042
58	1.786	1.837	1.815	0.195	0.194	0.237	1.591	1.643	1.578	1.604	0.034
55	0.289	0.247	0.255	0.290	0.291	0.301	-0.001	-0.044	-0.046	-0.030	0.025
56	3.244	3.215	3.013	0.254	0.257	0.250	2.990	2.958	2.763	2.904	0.123
65	3.328	3.387	3.201	0.323	0.303	0.300	3.005	3.084	2.901	2.997	0.092
20	1.676	1.650	1.656	0.158	0.157	0.201	1.518	1.493	1.455	1.489	0.032
16	1.742	1.769	1.693	0.205	0.205	0.241	1.537	1.564	1.452	1.518	0.058
27	0.973	0.825	0.789	0.371	0.387	0.406	0.602	0.438	0.383	0.474	0.114
51	1.525	1.484	1.560	0.164	0.156	0.212	1.361	1.328	1.348	1.346	0.017
30	1.997	2.009	1.950	0.524	0.526	0.611	1.473	1.483	1.339	1.432	0.080
52	0.860	0.777	0.738	0.172	0.163	0.170	0.688	0.614	0.568	0.623	0.061
54	2.481	2.268	2.149	0.183	0.181	0.190	2.298	2.087	1.959	2.115	0.171
29	2.750	2.851	2.772	0.268	0.319	0.318	2.482	2.532	2.454	2.489	0.040
50	0.958	0.874	0.782	0.131	0.128	0.138	0.827	0.746	0.644	0.739	0.092
33	2.140	2.237	2.100	1.489	1.505	1.619	0.651	0.732	0.481	0.621	0.128
28	0.786	0.804	0.794	0.135	0.132	0.159	0.651	0.672	0.635	0.653	0.019
43	3.301	3.387	3.071	0.263	0.267	0.278	3.038	3.120	2.793	2.984	0.170
22	0.571	0.561	0.587	0.149	0.154	0.166	0.422	0.407	0.421	0.417	0.008
60	0.798	0.804	0.784	0.137	0.136	0.155	0.661	0.668	0.629	0.653	0.021
24	3.119	3.027	2.821	0.302	0.287	0.291	2.817	2.740	2.530	2.696	0.149
61	1.636	1.690	1.652	0.351	0.350	0.402	1.285	1.340	1.250	1.292	0.045
41	1.278	1.212	1.223	0.344	0.346	0.410	0.934	0.866	0.813	0.871	0.061
35	1.549	1.466	1.460	0.119	0.108	0.119	1.430	1.358	1.341	1.376	0.047
44	0.619	0.681	0.547	0.324	0.332	0.334	0.295	0.349	0.213	0.286	0.068
46	1.688	1.484	1.467	0.170	0.167	0.166	1.518	1.317	1.301	1.379	0.121
18	0.716	0.522	0.534	0.114	0.113	0.125	0.602	0.409	0.409	0.473	0.111
17	0.224	0.231	0.233	0.125	0.126	0.157	0.099	0.105	0.076	0.093	0.015

48	1.342	1.139	1.096	0.235	0.221	0.212	1.107	0.918	0.884	0.970	0.120
2	0.764	0.766	0.762	0.197	0.189	0.221	0.567	0.577	0.541	0.562	0.019
25	0.778	0.581	0.590	0.441	0.449	0.439	0.337	0.132	0.151	0.207	0.113
26	0.053	0.060	0.068	0.059	0.060	0.061	-0.006	0.000	0.007	0.000	0.007
45	0.481	0.395	0.382	0.155	0.159	0.169	0.326	0.236	0.213	0.258	0.060
53	3.108	3.137	2.830	0.203	0.201	0.207	2.905	2.936	2.623	2.821	0.172
40	0.499	0.453	0.446	0.137	0.131	0.138	0.362	0.322	0.308	0.331	0.028
59	0.494	0.451	0.436	0.099	0.097	0.108	0.395	0.354	0.328	0.359	0.034
49	0.385	0.275	0.315	0.169	0.166	0.171	0.216	0.109	0.144	0.156	0.055
32	0.646	0.433	0.383	0.304	0.329	0.307	0.342	0.104	0.076	0.174	0.146
47	0.490	0.457	0.490	0.186	0.186	0.230	0.304	0.271	0.260	0.278	0.023

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