Strain variation of Respiratory Syncytial Virus in Qatar and its relationship to B-cell epitopes from the attachment (G) protein of RSV (B) strain.

By

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I would like to dedicate this thesis to my family

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Abstract

Respiratory Syncytial Virus (RSV) is the major cause of acute lower tract infection in early childhood. Annual epidemics occur which are well documented in developed countries during winter months, placing considerable pressure on the provision of health care. Little is known about the epidemiology of RSV infection in the Middle East and other desert climate regions of the world.

The aim of this project was to study the specificity of the immune response to RSV B in children, and to relate this to the infecting RSV strain, with particular emphasis on antibody response to RSV attachment (G) protein.

RSV is an important cause of hospital admission in children (54%) during winter months in Qatar. 63% of these infections are due to RSV A. A case study involving analysis of RSV strains from hospitalized children was carried out in Qatar over 2 winter seasons (1999-2000 and 2000-2001). RSV incidence was found to correlate with high relative humidity and low temperature. A comparison of enzyme immuno assay (EIA) and polymerase chain reaction (PCR) for the detection of RSV in clinical samples demonstrated that PCR was more sensitive than EIA. All positive RSV samples obtained during the study period were classified as belonging to RSV A and B subtypes using Multiplex PCR.

In this project, primer sets were designed and optimized to amplify the whole of the G gene of RSV A and B strains. Derived sequence analysis allowed deduction of the molecular epidemiology of RSV G gene for RSV A and B strains in Qatar and elsewhere. Sequence data of the G gene from Qatar RSV A&B isolates confirmed the variability of this protein and showed that variability occurs among group B RSV

viruses isolated in Qatar (0.8%), although to a lesser extent than among the group A viruses (5%) from same location. However, the group B viruses isolated in Qatar were highly variable in G gene sequences compared to the prototype strain RSVB N2 (13%) and to strain world-wide (10%) at the nucleotide level.

In order to produce an epitope map of the RSVB G protein, synthetic peptides representing linear B-cell epitopes of a representative Qatar RSV B isolate (B/Q/28/00) were used. The reactivity of human sera with the synthetic peptides was studied using sera from young children from whom RSV had been isolated. The sera from these children had variable peptide binding responses against different regions of the G protein of RSV B and the responses were focused on the conserved region. The results indicated that peptide 14 (a. a 150-165) of the G protein, in the conserved region of the protein, is a major antigenic site. This peptide sequence was recognized by a majority of the tested sera (93% sera).

To examine the relationship between neutralization antibody titre and reactivity to the synthetic peptides in children's sera, a modified micro-neutralization method was used. There was no significant correlation between the peptide binding activity in the sera and the neutralization titre of these sera. Sera from children infected with RSV A bound to peptides from the G protein of RSV B strain. However, children infected with RSV B had greater neutralizing antibody titre to RSV B strain than to the two RSV A strains. There was no difference in the neutralization antibody titre in sera to RSV A when assessed with prototype strains isolated many years ago (RSV A2), and with the recently-isolated strain A/Q/10/00.

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LIST OF ABBREVIATIONS

°C	Degree centigrade.
μl	Microliters.
ALRI	Acute lower respiratory tract infection.
APS	Ammonium per sulphate
BAL	Broncioalveolar lavage
BBG2Na	The peptide from the G glycoprotein conjugated to the albumin-binding domain of streptococcal protein G
bp	Base pairs
BRSV	Bovine Respiratory syncytial virus
BSA-PBS	Bovine Serum Albumin/phosphate buffered saline
C- terminal region	Carboxyl-terminal region.
CDRs	Complementarity determining regions
CO₂	Carbon dioxide.
cp	Cold passage
CPE	Cytopathic effect.
CTM	Cytotoxic T cells.
CX3CL	The chemokine fractalkine
deg./kt	Degree from true north/knots
DNA	Deoxyribonucleic acid.
EDTA	EthyleneDiamineTetraacetic Acid
EIA	Enzyme immune assay (Rapid antigen detection test).
EIMFA	Enzyme immuno membrane filter assay
ELISA	Enzyme linked immunosorbent assay.

ETCC	European tissue culture collection.
F	Fusion protein of RSV.
FCS	Fetal calf serum
FDA	The Food and Drug Administration
Flu	Influenza virus.
G	Attachment glycoprotein of RSV.
GAGS	Glycosaminoglycans
gp	glycoprotein
GPV	Global programme for Vaccination.
HA	Heamagglutinin.
HbsAg	The surface antigen of hepatitis B virus
Hep-2	Human epidermoid larynx carcinoma (epithelial cells).
HF	Hydrofluoric acid
HIS	Health information system
HIS	Health information system.
HIV	Human immunodeficiency virus
HMC	Hamad medical corporation.
HMPV	Human metapneumovirus
HPA	Health Protection Agency
HPLC	High performance liquid chromatography
hr	Hour
HRSV	Human Respiratory syncytial virus
ICAM-1	Intercellular adhesion molecule-1
IFA	Immuno fluorescence antibody
IIF	Indirect Immuno fluorescence

Km	Kilometre.
l	Litre
L	Viral polyprotein of RSV.
LRI	Lower respiratory infection.
LSHTM	London School of Hygiene and Tropical Medicine
M	Matrix protein of RSV.
MEDI-493	Humanizing mouse anti RSV antibodies
MEM	Minimal essential medium.
MHC	Major histocompatibility complex.
min	Minute.
ml	Milliter.
mM	Millimolar/millimole.
N	Nucleoprotein of RSV.
No.	Number.
NPA	Nasopharyngeal aspirate.
P	Phosphoprotein of RSV.
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate buffer saline.
PBST	Phosphate buffer solution /Tween
PCR	A nested multiplex polymerase chain reaction.
PFP	Purified fusion protein
RAMP	Rapid multiple peptide synthesis.
RANTES	regulated on activation, normal T cell expressed and presumably secreted
RNA	Ribonucleic acid

rpm	Revolutions per minute.
RSV	Respiratory syncytial virus.
RT	Reverse transcriptase.
RTI	Respiratory tract illnesses
SD	Standard deviation
sec	Second.
SH	Small hydrophobic protein.
Sq	Square.
Ta	Annealing temperature.
Taq	DNA polymerase of thermophilic bacterium *Thermus aquatics*.
TEMED	N,N,N,N – Tetra methylethylenediamie
Temp	Temperature
TFA	Trifluoroacetic acid
Tris-HCl	Tris (hydroxymethyl) aminomethane hydrochloride.
UV	Ultra Violet
WHO	World health organisation.
Wt	Wild type

1. Chapter 1: General Introduction

Virology

1.1 Background

Respiratory disease is a common cause of morbidity and mortality in the general population, although the most severe illness is often seen among infants and the elderly. Several viruses can cause respiratory illnesses, the resulting symptoms of which include fever, coryza, otitis media, laryngitis and bronchiolitis. It is difficult to distinguish between different causal agents since similar symptoms are often seen, so to be certain laboratory diagnosis is necessary. The most common viral respiratory pathogens causing lower and upper respiratory tract infections are influenza and respiratory syncytial virus (RSV).

Human respiratory syncytial virus (HRSV) causes respiratory tract infections particularly in infants and young children. There is also increasing evidence that points to RSV causing significant disease in the elderly (Falsey et al., 1995). The infection can vary in severity ranging from a mild infection, similar to a common cold, to severe pneumonitis or bronchiolitis in susceptible individuals. RSV infection is estimated to cause 90,000 hospitalizations and 4,500 deaths per annum in the USA in children and huge economic implications, costing an estimated $300 million in the USA (Meissner, 1994). Approximately 18, 000 children annually (predominantly infants) are admitted to hospital with diagnosis of bronchiolitis in the UK, with 80% caused by RSV infection (Crowcroft et al., 1999). In urban areas of Britain, 2% of infants need hospitalization as aresult of RSV infections (Sims et al., 1976)

Little is known about the epidemiology of RSV infection in developing and Middle Eastern countries. In most studies, RSV was found to be the predominant viral cause of acute lower respiratory tract infections (ALRI) in childhood, being responsible for 27-96% (mean 65%) of hospitalized cases in which a virus was found in the Gambia (Weber et al., 1998a). RSV infection is

seasonal in most countries (Stott & Taylor, 1985). Very little information is available about the incidence and mortality of children infected with RSV or strain variation of circulating strains. Further studies on RSV should address these questions in more detail. The availability of information on the molecular epidemiology and seasonality of these infections from this part of the world would be important in planning vaccination and treatment strategies.

1.2 History of RSV

RSV was first isolated in 1956 from a laboratory chimpanzee with respiratory symptoms (Blount et al., 1956). Soon after, the virus was isolated from a child with pneumonia in Baltimore and the characteristic syncytial cytopathic effect was observed, hence its name (Chanock & Finberg, 1957). RSV was soon recognized as an important cause of respiratory illness across the globe (Anderson et al., 1991).

1.3 Taxonomy

RSV is classified in the order *Mononegavirales*, which consists of four families designated by the International Committee of Taxonomy of Viruses. The family *Mononegavirales* also includes the *Rhabdoviridae, Filoviridae,* the *Bornaviridae* and the *Paramyxoviridae. Mononegavirales* is derived from the Greek word *monos* meaning single, *nega* from negative and *virales (*from the Latin meaning viruses). All members have a linear negative stranded monopartite genome with a similar genome arrangement (3' non-translated region-env-pol-5' non-translated region). They also have a helical nucleocapsid, initiation of translation by a virion associated RNA dependent RNA polymerase from a single 3' promoter (Murphy, 1995) RSV is a member of the *Paramyxoviridae, which* is divided into two sub-families; the *Paramyxovirinae* and the *Pneumovirinae* All members of the family possess an envelope, are large in diameter, approximately 150 nm and are

pleiomorphic. They have a non-rigid helical nucleocapsid that is negative sense. The virion has a lipid bi-layer derived from the host and has 2-3 glycoprotein projections, 13-18nm long, spaced 7-10 nm apart, depending on the genus.

The *Paramyxoviridae* possess three nucleocapsid-associated proteins, RNA binding protein (N or NP), a phosphoprotein (P) and a large polymerase protein (L). The L and P proteins form the viral polymerase. They also have three membrane-associated proteins, non-glycosylated envelope protein (M) and two glycosylated proteins, the fusion (F) and the attachment protein (G, H or I-IN). The pneumoviruses, including RSV, are in their own sub-family because they do not have the same gene order and are not related antigenically to members of the *Paramyxovirinae* *(Fig. 1.1)*.

1.4   Viral genome

The RSV genome is 15,222 bp in the prototype strain A2 in length consisting of 10 genes encoding 11 major proteins **(Fig 1.2)** There are three transmembrane proteins, F, G and SH, two matrix proteins, Ml and M2. There are also structural proteins, N, P and L and three non-structural proteins, NS1, NS2 and M2-2. There are intergenic regions consisting of a few nucleotides that are scanned by the polymerase, but not transcribed.

Figure 1.1: Taxonomic Tree of Respiratory Syncytial virus

Order

↓

Mononegavirales

↓

Family

↓

Paramyxoviridae

↓

Sub-family

↓

Pneumovirinae

↓

Genus

↓

Pneumovirus

↓

Species

| Human respiratory Syncytial virus (HRSV) | Bovine respiratory Syncytial virus (BRSV) | Ovine respiratory Syncytial virus (BRSV) |
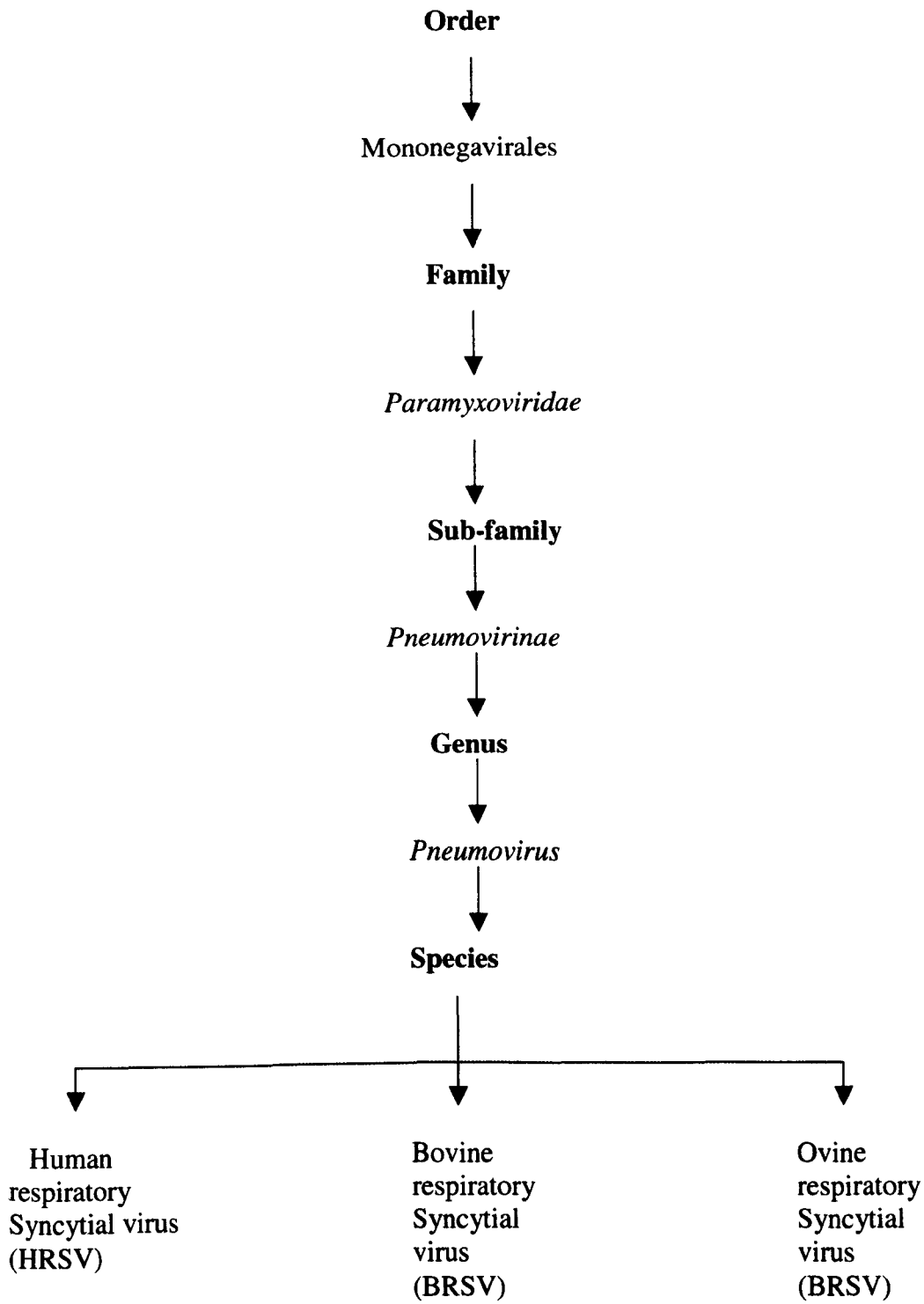
Figure 1.2 (a): RSV gene order.
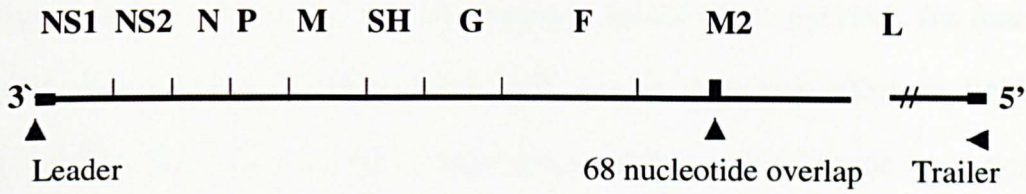
Genes & nucleotide length (bp):

NS1 532, **NS2** 503, **N** 1203, **P** 914, **M** 958, **SH** 410, **G** 923, **F** 1903, **M2** 961, **L** 6578.

Figure 1.2 (b): Diagrammatic representation of RSV virion.
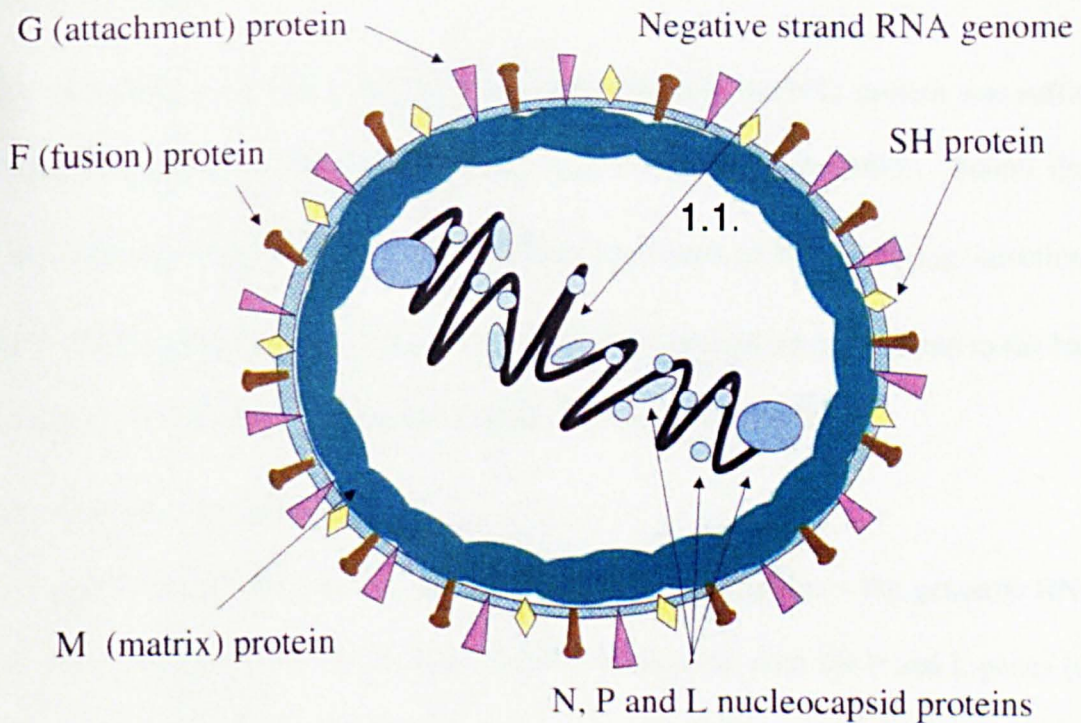
1.5 Viral proteins

1.5.1 Non-structural proteins

Separate mRNAs encode the two non-structural proteins NS1 and NS2. The function of the proteins is not known, but it is believed that NS1 has an inhibitory effect on RNA transcription and replication (Atreya et al., 1998). The proteins may be involved in the regulation of the other RSV genes or aid transportation of viral proteins to the cell membrane (Teng & Collins, 1999).

Recently, a key genetic component determining the ability of highly virulent viruses (e.g. influenza type H5N1) to resist the inhibitory effects of host cytokines such as interferon and tumor necrosis factor α, seems to be the non-structural protein of the virus, NS1. Although small, this protein already has many assigned functions and has been pinpointed as the major tool that influenza uses to avoid switching on host innate immunity (Zambon & Barclay, 2002; Seo et al., 2002; Garcia-Sastre et al., 1998).

Seo et al (2002) found that a single amino acid change in the NS1 protein was sufficient to confer cytokine resistance in their animal model system. Poole et al (2002). Found that the NS1 of paramyxoviruses may play a role on limiting the yield of IFN-γ during infection as a general property of paramyxoviruses. This indicates that NS1 protein can play a role in the balance between viral replication and the host immune response (Poole et al., 2002).

1.5.2 Structural proteins

The N gene encodes the nucleocapsid protein, which encapsulates the genomic RNA to form the nucleocapsid template, and is involved in RNA replication, with the P and L genes (Grosfeld et al., 1995). The N gene binds tightly to genomic and antigenomic RNA to form the ribonucleoprotein template. The N gene is highly conserved within subtypes (Johnson & Collins, 1989) and therefore

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is the target for study of the classification and sub-typing of strains of RSV (Cane & Pringle, 1991).

The P gene encodes the major phosphoprotein which has two functions, first as a transcription factor and second as a polymerase cofactor (Barik et al., 1995).

The L gene product is the polymerase. The M2 and the L genes overlap by 68 nucleotides. The L protein is never found free in the infected cell, but forms a complex with P to constitute the viral polymerase (Hamaguchi et al., 1983).

1.5.3 Matrix proteins

There are two matrix proteins, both of which are non-glycosylated. The predominant matrix protein, Ml is the most abundant in the virion and its function is to transcriptionally inactivate the nucleocapsid. A second function is to assist in the interaction of the nucleocapsid and the envelope (Ulloa et al., 1998).

The second matrix protein, M2 is only present in the *Pneumovirinae*. This protein is important in virus transcription and balances RNA replication and transcription (Bermingham & Collins, 1999). M2 is a processivity factor for mRNA transcription that enhances transcription readthrough at intergenic regions and is a transcriptional antiterminator (Hardy & Wertz, 1998).

1.5.4 Surface glycoproteins

The F gene encodes a transmembrane surface glycoprotein necessary for fusion of the virus to cell surface membranes and mediates virus penetration. It is also responsible for the formation of syncytia from infected cells. The F protein is more conserved than the G protein, and is a major antigenic protein. It consists of two subunits, the F1 and F2 linked by a disulphide bridge (Lopez et al., 1998).

The G gene is 923 nucleotides in length and encodes the attachment protein (inferred because antibodies specific for G inhibit virus binding to receptor (Levine et al., 1987). It is a type II glycoprotein of 289-299 amino acids with a single hydrophobic domain and an N terminal single

transmembrane region. The G protein precursor is 33 Kd, which is subjected to post translational modification by the addition of N and O linked carbohydrate side chains to produce a mature protein. The protein has a very high serine, threonine and proline content which is analogous to mucin proteins.

The protein consists of three regions, the intracellular, transmembrane and extracellular. The extracellular region is separated into two variable regions separated by a conserved region (Cane, 1993) **(Fig 1.3).** The conserved region is thought to be involved in cell receptor binding, as it is believed to be conserved between subgroups and genotypes.

More recently a highly conserved domain of G has been shown to bear homology to the chemokine fractalkine (CX3CL). In vitro interaction of G and the fractalkine receptor (CX3CR1) has been demonstrated, and pre-treatment of cells with fractalkine has been shown to inhibit viral plaque formation. Virtual abolition of viral plaque formation is achieved by pre-treatment of cells with both heparin and fractalkine (Tripp et al., 2001). Interestingly, it has long been known that RSV infected cells produce a secreted form of G, and it was previously believed that this was essentially a decoy for anti-G protein antibody. The homology with CX3CL raises the possibility of G having a role in immune subversion, by mimicking CX3CL.

Figure 1.3: G protein structure

a)

| 1 | 50 | 100 | 150 | 200 | 250 | 298 |

b) N   AUG   64G   021/1G 021/2G **1   2   3   4** 63GC

conserved   variable   conserved   variable

c)
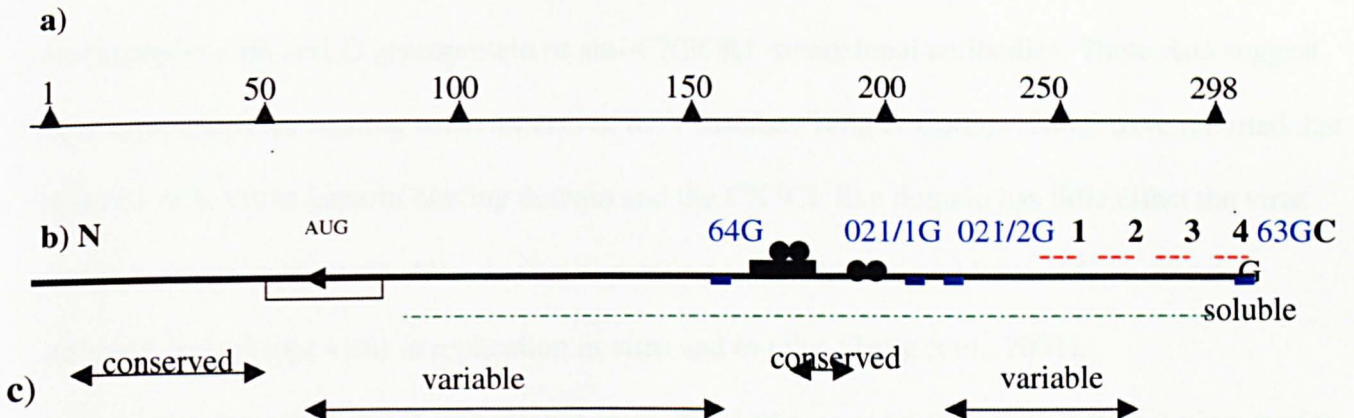
Linear representation of G protein. **a)** Amino acid number. **b)** The protein is shown as the dark bar, with N and C terminal indicated. The white box is the transmembrane domain. Cysteine residues are marked with black dots. The black box indicates the strictly conserved 13 amino acid central region. Red dotted lines represent the position of synthetic peptides recognized by human convalescent sera, numbers corresponding to amino acid 1=229-243, 2=247-258, 3=262-276 and 4=280-294 respectively. The small blue bars represent the epitopes, which afford maximal neutralization. The G soluble protein is shown with the green dash dot line and the initiation codon of this segment with AUG. **c)** the variable and conserved regions of G protein.

These data suggest the importance of the putative heparin binding domain and the CX3C-like domain. In addition, Tripp et al., (2003) have shown that RSV G glycoprotein reduces respiratory rates associated with the induction of G glycoprotein-CX3CR1 interaction, an effect that is inhibited by treatment with anti-G glycoprotein or anti-CX3CR1 monoclonal antibodies. These data suggest new approaches for treating some aspects of RSV disease. Teng & Collins (2002) have reported that deletion of both the heparin binding domain and the CX3CL like domain has little effect the virus attachment and replication. Also, a virus expressing solely the secreted form of G is almost as efficient as wild type virus at replication in vitro and in mice (Teng et al., 2001).

After testing the reactivity of monoclonal antibodies and escape mutants with different virus strains, three types of epitopes have been identified. The first type is conserved; the second is group-specific shared by all viruses of the same antigenic group. The third is strain specific/variable and is present in certain isolates of the same antigenic group (Martinez et al., 1997). The conserved region is located between positions 164-176 that includes 4 conserved cysteine residues at positions 173, 176, 182 and 186. The conserved region has a subgroup-specific immunodominant site at 174-188. The studies showed within the amino acid sequence 174-187 are the sub-group specific protective epitopes.

A synthetic peptide from the same region containing a Cys-Ser substitution at position 186 completely protects immunized mice against group A RSV infection (Simard et al., 1997). This study also showed that amino acids 125-203 of subgroup A glycoproteins represent a domain which is fully protective and completely prevents replication of RSV A in mice.

Within G gene sequence variation of approximately 47% between the A and B subgroups has been shown and the most extensive amino acid sequence differences between groups are found on the G gene (Johnson et al., 1987).

The **SH** gene encodes a small hydrophobic glycosylated protein with unknown function (Bukreyev et al., 1997).

Separating the genes are conserved intergenic regions, which are not copied into the mRNA and they vary between 1-56 nucleotides in length.

1.6   Replication cycle

The viral attachment receptor is unknown at present. However, the conserved 13 amino acid segment in the middle of the G protein ectodomain (amino acids 164-176) is believed to include the putative receptor binding site (Johnson et al., 1987). Pre-incubation of RSV with heparin inhibits infection of cultured cells and the RSV attachment protein G was able to bind specifically to heparin (Krusat & Streckert, 1997). This study showed that heparin or other cell surface glycosaminoglycans (GAGS) are involved in RSV binding to cells through glycoprotein G.

A recent study by Melero and Martinez (2000) confirmed previous findings using flow cytometry to measure binding of RSV to cultured cells. It was shown that cell surface proteoglycans are important for RSV binding and that removal or destruction of GAG inhibits binding. However, there are other possible methods of attachment and entry to cells with different cell types. This has been shown by the competent replication of a mutant virus lacking the G gene in Vero cells, although impairment of growth occurred *in vivo* and in other cell lines. It is therefore thought that the interaction of RSV with the cell surface GAGs promotes attachment of the G gene to an unidentified receptor through a central conserved region (Karron et al., 1997). *In vivo* ciliated epithelium cells are peripherally infected. Therefore, it is possible that there is more than one mechanism of entry.

Virus entry occurs by fusion of the virus envelope with the cell surface membrane at pH7 and the protein involved is the fusion (F) glycoprotein. F is formed from a precursor Fo that becomes

activated after cleavage by a host protease to form virion disulphide linked Fl and F2 sub-units. The viral envelope becomes incorporated in to the host cell surface and the nucleocapsid is released in to the cytoplasm (Karron et al., 1997; Lopez et al., 1998).

The mechanism of replication has not been completely elucidated, but it is believed that the polymerase molecule binds to the 3' end of the RNA. The polymerase transcribes the RNA until it meets a gene end signal, then falls off and binds again at the next gene downstream. It has been shown that RSV has read through transcription (Hardy & Wertz, 1998). Here, the RNA polymerase does not fall off the RNA at a gene end signal, but carries on to the next gene, therefore enhancing production of genes further downstream. Replication involves the synthesis of a full length, positive sense copy (antigenome). This is found only in nucleocapsid form and is the template for synthesis of progeny genome.

Viral replication occurs in the cytoplasm and progeny virions form when nucleocapsids bud through the plasma membrane acquiring a lipid envelope containing virally encoded transmembrane proteins. RSV buds through the apical
surface that may contribute to the localisation of the virus to the respiratory tract as the virus buds into the tract (Roberts et al., 1994). The cytopathic effect induced by the virus causes syncytia formation, which leads to cell death.

1.7   Strain analysis

Strain analysis is important because it provides information about epidemiology and transmission of RSV (Anderson et al., 1991). On the basis of monoclonal antibody studies, RSV has been classified into two major groups A and B, and within these groups, antigenic subtypes of RSV have also been identified, differences between RSV strains have been observed with monoclonal antibodies specific for the nucleoprotein (NP) component, matrix protein (M), large glycoprotein (G) and

fusion protein (F) (Mufson et al., 1985). Sequence studies of the nucleoprotein (N), phosphoprotein (P), small hydrophobic (SH) protein and attachment (G) protein gene have confirmed the division of RSV into two groups and also identified numerous variants or lineages within each group (Peret et al., 1998). The RSV groups A and B circulate indepented in the population with group A being somewhat more prevalent (Cane et al., 1992).

Most molecular studies involving variation have concentrated on the N, P and SH genes using restriction enzyme analysis of PCR amplicons (Cane & Pringle, 1991). Six lineages or genotypes based on restriction fragment length polymorphism (RFLP) analysis of the SH gene have been identified for subgroup A (Cane et al., 1994). Subgroup B has been divided into two lineages using the N and P region of the genome and into six lineages using the G gene (Peret et al., 1998). RSV strain variation occurs because of the variation seen within the G gene and this variation probably results in the ability of RSV to re-infect individuals. The G protein is involved in neutralization and protective antibody responses. There are a high percentage of nucleotide changes resulting in amino acid changes, which could cause a selective advantage to G protein changes (Cane & Pringle, 1995b). There is variation of approximately 47% within the G gene between the two subgroups (Johnson et al., 1987) and intrasubgroup variation is approximately 20% for group A strains (Cane & Pringle, 1995b) and 9% within group B (Sullender et al., 1991; Cane &Pringle, 1995; Sullender et al., 1998). A more recent study has suggested that variation in the G protein does not correlate with severity of infection (Brandenburg et al., 2000b).

Genetically similar strains cluster temporally rather than by geographical location, suggesting immunological pressure may determine the genetic variation and evolution of RSV from the same antigenic group. However, this information is limited and requires further analysis by antigenic characterization of clinical isolates and sequence information (Cristina et al., 1991). Knowledge of humoral responses to known genotypes will enable strain variation to be understood more clearly.

Multiple strains circulate simultaneously in the same community and different strains circulate in different communities during the same year. A study by Cane between 1988 and 1993 in Birmingham showed yearly variation in the predominating circulating RSV strain, and it was suggested that G gene variation confers an advantage to the virus allowing re-infection (Cane et al., 1994), this study showed some lineages of subgroup A increased in number over several years and then decline possibly because of increased resistance in the community to a particular genotype. A study based in the Gambia assessed the prevalence of RSV over four epidemics (Cane et al., 1999). This study also showed that variation in the predominating sub-types was observed between seasons. This was considered likely to represent evolutionary changes within the virus (Cane et al., 1994; Garcia et al., 1994). Studies in Korea also showed that multiple genotypes co-circulate in each epidemic and a shift occurred in the predominant genotypes of G gene for subgroup A viruses over 9 consecutive epidemics (Choi & Lee, 2000). Another study done over two seasons in several states in USA showed that RSV strains co-circulate (Anderson et al., 1991). In this study, however, differences in the predominant subgroup were seen between the different States in the same epidemic, which suggested that the same predominant strains were not circulating throughout the country at the same time.

A study in Denmark over three epidemics showed a pattern of predominance in the circulating strains. Other studies performed over a five year period in the Urgway showed a pattern of predominance of certain strains with no one strain predominating more than once in five years (Cristina et al., 1991). Speculation has arisen that variation in the predominant strain was because the novel strain can evade the immune system and therefore circulate freely. However, as most research has been based on a small sample number and only on hospitalized infants, it is difficult to understand the basis of the circulating strains in the whole community.

There is little information available about the burden of disease in developing countries. A study in Mozambique showed a higher prevalence of sub-group B viruses. Most of the strains circulating in

a single outbreak isolated in very distant places in different years were more genetically linked than strains co-circulating in the same group isolated during the same outbreak in the same geographical region. However, there was one cluster more genetically distant from previous isolates found in other regions (Roca et al., 2001).

One possible model, which has been elucidated to explain antigenic variation, is that immunological pressure in a community forces positive selection towards new antigenic variants of RSV and therefore accounts for the ability to re-infect. This is supported by antigenic changes in the G protein and amino acid changes, which accumulate progressively. Evidence of co-circulating strains present in the community in low frequency may proceed a season of dominance (Cane et al., 1994). The disappearance of a strain may be due partly to increased herd immunity to that particular strain or because of increased resistance to a particular genotype (Johansen et al., 1997). There are a variety of possible mechanisms, which could cause changes within the G gene sequence. One mechanism is frame shifting which occurs by the deletion of a base resulting in a change of reading frames, which alters the production of the amino acids (Garcia-Barreno et al., 1990). It is likely that frame shifting occurs because of an error by the polymerase, which allows the virus to escape neutralization by monoclonal antibodies (Cane, 1993). Other mechanisms could include premature stop codons, substitutions and hyper-mutations (Sullender & Edwards, 1999).

RSV associated disease.

1.8   RSV epidemiology

RSV is a global disease that is highly infectious. A single exposure to an RSV outbreak gives a 50 - 100% risk of contracting disease, dependent on the setting. RSV is the most common microbial cause of pneumonia and bronchiolitis in children under one year of age, with one in one hundred children in the USA being hospitalised as a result of RSV infection. In one thirteen year study of

hospital admissions in Washington D.C., 43% of all infant bronchiolitis and 25% of infant pneumonia were caused by RSV. Although the death rate from RSV in developed countries has been falling, due largely to better intensive care treatment and not to any effective anti-viral therapy, it is estimated that between 0.3 and 1% of those admitted to hospital with RSV related disease will die. Risk factors for hospitalisation include low socio-economic status and living in heavily industrialised areas. Morbidity and mortality rates are highest in those with bronchopulmonary dysplasia or congenital heart disease, and those who are immunosuppressed in some way. As there are a number of agents that can cause RSV-like symptoms, the epidemiology of the virus in the developing world is more obscure (Brandt et al., 1973; Kim et al., 1973; Parrott et al., 1973; Collins, 2001).

Identification of two virus groups (RSV -A and -B) has led to studies to investigate the relationship between strain and disease severity. Several studies have found RSV-A to be more severe and others have shown no difference. Only two studies have shown RSV-B to cause more severe disease. These two groups co-circulate and the immune responses to each are mostly cross-reactive (Hall et al., 1990; Brandenburg et al., 2001). In areas of temperate climate, an 'RSV season' occurs. This lasts from late autumn, peaks in mid-winter, and ends in early spring. Although the timing of the outbreak is predictable, its severity is not (Hall et al., 1990; Brandenburg et al., 2001).

As no long-term protection immunity to RSV develops, it is increasingly recognized as an important disease in the elderly, causing approximately a quarter of acute respiratory illness in this group. It has been suggested that this group alone represent sufficient economic justification for the development of a vaccine (Falsey, 1998; Han et al., 1999).

1.9   Reinfection with RSV

Repeated infection with RSV throughout the life of an individual is common, even in the presence of moderate levels of RSV specific antibody (Hall et al., 1991). At two years of age, 50% of

children show signs of having had more than one RSV infection. Symptoms upon re-infection in both children and adults tend to be less severe. This may be due to protective immune mechanisms and/or the fact that re-infection must occur at a later age which will itself improve the outcome of infection.

Other respiratory viruses, such as influenza, re-infect but this is facilitated by antigenic change. How RSV reinfects so readily is yet to be defined but a number of factors are thought to be important. RSV is readily transmissible and replicates rapidly at site of infection.

This rapid replication is likely to outpace the generation of secondary immune responses especially CD8 lymphocytes. The secretory IgA response protecting the respiratory surface is short lived, while serum IgG has limited protective effect. These factors are probably important in allowing re-infection, but are shared with influenza virus. RSV's unique G protein may be important. It has an unusual non-globular structure, with multiple glycosylations, that may induce poor quality neutralising antibody and limited CD8 T-cell responses. G-protein is secreted by RSV infected cells during natural infection and may act as an antibody decoy and/or a CX3CL chemokine mimic hence subverting the immune response (Collins & Pollard, 2002).

Other possibilities of multiple infections with RSV occur through an individual's life because of the highly infectious nature of the virus (Blydt-Hansen et al., 1999). This is probably due to selection pressure and the virus escaping the immune system by changing antigenic regions and glycosylation sites. It is probable that the G gene shows extensive variability for this reason. Cytoplasmic and transmembrane regions are followed by a sharply defined hypervariable domain between amino acid 101 and 133. There is a more extended variable region in the carboxy terminal third made of three smaller variable regions. There are two major variable regions separated by highly conserved domains between 147 and 207. As most of the nucleotide changes in parts of the gene coding for the variable area result in coding changes, there may be immunological pressure for change in certain areas of the G protein and this may account for the ability of the virus to re-infect repeatedly

(Cane et al., 1991; Melero et al., 1997). A study by Sullender et al (1998) showed isolation of two strains of RSV A with *15%* amino acid variation from sequential infections in children.

The other possible reason that re-infection can occur is because the immune response upon infection is not protective. Re-infection could occur with a different virus, which is not recognized by the immune system. Studies have already shown that infection with one subtype does not confer immunity against another subtype or the same subtype (Mufson et al., 1987).

Re-infections may occur because of antigenic diversity and less effective, short lived immunity at mucosal site of replication. RSV is also resistant to effective neutralizing antibodies possibly mediated by the unique structure of the G gene, decreased immune response following RSV infection in the neonatal period due to immunological immaturity and immunosuppression mediated by maternally-acquired antibodies (Collins et al., 1999b).

1.10 Pathogenesis

Transmission of RSV occurs either directly by aerosols or fomites. After initial infection with RSV, the incubation period is approximately three to five days (Domachowske & Rosenberg, 1999). Virus replication commences in the nasopharynx, and then travels to the cells in the respiratory epithelium. RSV spreads to other uninfected cells by causing syncytia formation.

RSV causes a spectrum of disease symptoms and pathologies that are generally divided up according to which parts of the respiratory tract are infected- the most common symptoms due to RSV infection are simply the common cold. When only the upper respiratory tract is significantly infected, the patient gets rhinitis with a blocked and runny nose, and is generally irritable. This lasts a short time, days to a week, and requires no specialist treatment.

If infection occurs further down the respiratory tract in the bronchioles, the disease is more serious. It starts with common cold symptoms, and progresses 3 to 5 days later, to produce fever and symptoms due to difficulties in getting air out of the lungs; deep cough, breathlessness, rapid

breathing, expiratory wheezing and possibly cyanosis. In advanced cases the chest may become expanded due to increasing amounts of dead air space in the lung.

When infection occurs lower down the respiratory tract pneumonia occurs. The symptoms are similar to those mentioned above, but the expiratory wheeze is lessened because of obstruction of air coming from the alveoli due to narrowed bronchioles. Effective hospital care, possibly including supplementary oxygen, has reduced mortality rates to <1% in developed countries. Death if it occurs, is usually due to blockage of the airways via a number of mechanisms. Inflammation of the mucosa around the airways; copious mucus secretions in the lung lumen, the clearance of which is often impaired due to a breakdown in ciliary transport, caused by direct viral killing of mucosal cells, and the deposition of necrotic cells into the lumen of the lung. In RSV pneumonia, breathing is impaired by thickening of the alveolar walls, due to mononuclear cell infiltration, and a filling up of the alveolar lumen with copious amounts of mucus (Pu et al., 2001). The main reason for the increased susceptibility of babies to severe RSV-related disease is thought to be mechanical with their narrower airways being more susceptible to blockage. Although much of RSV pathology is caused by inflammatory mechanisms, anti-inflammatory drugs such as corticosteroids are not an effective therapy (Brandenburg et al., 2001).

1.11 RSV and Asthma

Correlation of severe RSV associated disease and asthma has been conclusively demonstrated (Bont et al., 2000; Bont et al, 2001; Sigurs et al., 2000) . Studies in a mouse model of RSV and asthma have demonstrated that airway hyper-responsiveness to either dust mite faecal antigen or ovalbumin can be induced by prior RSV infection, and that this allergic airway sensitization may be transferred by T lymphocytes. Schwarze et al demonstrated that both CD8 cells and IL-5 were necessary for the development of this hyper responsiveness in mice. These data suggest that severe RSV infection in humans may have a causal effect in the development of asthma in later life (Schwarze et al., 1999 a;

Schwarze et al., 1999 b). Therefore, if this causative link is true, prevention of severe RSV disease in infants, using a vaccine, should have a beneficial effect on subsequent asthma rates.

1.12   RSV subgroups and their clinical importance

RSV is divided into two subgroups A and B based on studies using monoclonal antibodies (Anderson et al., 1985; Belshe et al., 1982b) . RSV A seems to be more prevalent than RSV B. Infection with one subtype does not prevent future infection with the other subtype. It is thought that RSV A causes a more severe disease (McConnochie et al., 1990; Walsh et al., 1997) . However, in another study RSV B was found to cause a more severe disease using clinical and immunological markers (Hornsleth et al., 1998). A study in Argentina over seven consecutive epidemics showed that both strains co-circulated and that sub group A occurred at least eight times more often in six out of the seven years. Subgroup A was more common with bronchiolitis and subgroup B was more common with pneumonia (Carballal et al., 2000; McConnochie et al., 1990). However, as most studies have involved small numbers of predominantly hospitalized infants, there is little information relating to the severity in RSV infection in populations as a whole.

1.13 Laboratory Investigations

The clinical presentation of RSV is often similar to other respiratory tract infections and therefore the only method of a definitive diagnosis is the use of laboratory tests. Wheezing is a common indication of RSV infection in young children. Samples often include nose and throat swabs and nasopharygeal aspirates (NPA) and RSV can be detected by cell culture, using Hep 2 cells, which results in characteristic cytopathic effects (cpe) of syncytia if a sample is positive. However, problems with this method include difficulties in growing the virus and lack of cpe (especially seen with RSV B). Also, RSV is a labile virus and therefore needs to be stored at ~80° C to maintain viability. Other detection methods include EIA and immunofluorescence, which are specific,

sensitive and rapid.

Commercial kits are also available for diagnosis of RSV, including the Becton Dickinson Directigen rapid antigen test, which showed a sensitivity of 83% and a specificity of 90% when NPA samples were tested. The advantage of these kits is they produce results extremely quickly (within approximately 15 minutes). They are very easy to use, not requiring any special equipment, are read by eye and hence can be used within the hospital ward as a bedside kit (Kok et al., 1990). However, the tests are not widely used, as there is a dispute over sensitivity and specificity. Another kit also based on enzyme immunoassay methodology, Abbott Diagnostics Testpack, has independently been evaluated in conjunction with Directigen. The study showed that Directigen had a sensitivity of 76% whereas Testpack had a higher sensitivity of 92% (Rothbarth et al., 1991). A further study performed in Edinburgh showed the Directigen had a sensitivity of 79% and a specificity of 97% in comparison with direct immunofluorescence. Results showed that a positive result is trustworthy, not requiring laboratory confirmation, but a negative result does need confirmation (Mackenzie et al., 1999). The results of the study showed that it was necessary for the sensitivity of the commercial tests to be increased before they can be used routinely, but when sensitivity is improved they will be extremely useful in hospitals when choosing treatment or preventative measures (Borriello, 1999).

As a result of the advent of molecular methods for the detection of a variety of virus genomes, diagnosis can be achieved more quickly and is more sensitive. The advantage of molecular technology is that sequence information can also be obtained. Polymerase chain reaction (PCR) tests are often performed routinely on clinical samples and results can be obtained within 48 hours.

Host Response to RSV

1.14 Virus tropism

RSV enters the body via the respiratory tract and infection is restricted to the respiratory tract, affecting mainly the epithelial cell layer. The receptors for the virus include CX3CR (the fractalkine receptor), via the G-protein; extracellular sugars via with both G and F; and ICAM-1 which has been shown to bind to RSV F protein (Arnold & Konig, 1996; Behera et al., 2001). None of the known receptors satisfactorily explain the limited RSV cell tropism.

RT-PCR studies have shown RSV RNA to be present in peripheral blood mononuclear cells (PBMCs), although there is no evidence for RSV replication outside of the respiratory tract in immunocompetent individuals. In immunocompromised patients, the duration of disease increases and in some cases RSV spreads to other areas of the body (Milner et al., 1985).

A combination of cellular and humoral immunity is involved in combating RSV infection. Humoral immunity probably controls RSV infection. An infant may be protected by maternal antibody for only a couple of months. Breast fed infants may however, be protected for longer because of receiving antibody in colostrum (Mito et al., 1984). Cellular immunity is important in viral clearance and a recent study shows characterization of a novel HLA B7 restricted memory RSV specific CTL epitope in humans. The epitope lies within the NP gene, although strong RSV-specific CTL responses directed towards epitopes within SH, F, M and NS 1 in human RSV specific CTL activity have also been seen. Once dominant RSV specific CTL epitopes have been identified it will be possible to concentrate on the role of RSV specific CTLs in controlling disease in pediatric infection (Goulder et al., 2000).

Different proteins in the virus generate different immune responses. Immunity to F protein is cross-reactive, whereas immunity to G is usually group specific. The F protein activates a CD^4 Th1 response, causing production of IL-12 and interferon gamma. The G gene however, produces a Th 2

response, with production of IL-4 and 5, hence causing extensive eosinophilia. Recently, the region of the G gene responsible for this elevated production of eosionphils has been mapped to a region between amino acid 193-203 in the second variable region of the G gene (Sparer et al., 1998). There is also another region believed to be important in generating the humoral response. In one study they showed that if CD4$^+$ cells were deleted in Balb/c mice prior to administration of peptide from amino acid 184 to 198, eosinophilia did not occur, whereas the elimination of CD8$^+$ cells had no effect.

The CD8$^+$ cells are believed to regulate the CD4$^+$ response. In a study performed by Hussel using Balb/ c mice, which produce a CD4+ response to RSV G protein (Hussell et al., 1997), severe lung pathology due to acute eosinophilia was observed. When CD8+ cells were administered the disease was less severe, possibly shifting the immune response away from being Th2 mediated. Another study showed similar results with the probability that CD8$^+$ cells down regulate Th2 cytokine production and hence eosinophilia (Srikiatkhachorn & Braciale, 1997a). RSV infection may induce lung inflammation via early production of inflammatory chemokines. The presence of histamine, eosinophilic cationic protein and major basic protein in NPA and tracheobronchial aspirates show RSV infection triggers migration to the airways and activation of basophils and eosinophils. In the mouse model, the pathogenesis of RSV induced lower airway disease is similar to that in humans. In Balb/c mice, RSV rapidly replicates in lungs after intranasal inoculation and induces mononuclear cell infiltration. These changes correlate with the amount of viral inoculum. In human RSV, a more severe disease occurs in children who have higher concentration of RSV in their secretions. Chemokines are involved in RSV mediated lung inflammation and chemokines and MIP1 are involved in the pathogenesis of lung inflammation in mice experimentally infected with RSV (Haeberle et al., 2001).

Cytokines secreted during RSV infection include interleukin 8, which is a chemotactic chemokine, IL-6, 1L-10 and RANTES, which are also thought to regulate RSV infection (Sheeran et al., 1999).

Lung surfacant protein A increases attachment of RSV and therefore subsequently enhances entry into host cells (Hickling et al., 2000).

1.15 Cellular immune response

In vivo and in vitro RSV infection of respiratory epithelial cells leads to production and release of chemokines, notably RANTES, MCP-1, MIP-la and IL-8 (Olszewska et al., 2000; Thomas et al., 2000; Casola et al., 2001; Haeberle et al., 2001). This response leads to an inflammatory influx which, when measured in broncioalveolar lavage (BAL) fluids from infants with RSV bronchiolitis, is made up of neutrophils (~75%), monocytes (~10%) lymphocytes (~10%).

Monocytes recognise RSV as a pathogen using the pattern recognition receptors TLR-4 and CD 14, In vitro, RSV activates these receptors, causing cytokine release; in vivo prevention of the RSV TLR-4 interaction has been shown to reduce monocyte and NK cell trafficking into infected mouse lungs (Kurt-Jones et al., 2000; Haynes et al., 2001).

The T-lymphocyte response is critical to the successful resolution of infection. Children with deficient T-cell function, are unable to clear the virus for prolonged periods (Milner et al., 1985). Also severely immunocompromised adults (e.g. those receiving bone marrow transplants) have a high incidence of RSV infection leading to serious disease, even death (Collins, 2001).

In Balb/c mice infected with RSV, passive transfer of RSV specific T-cells is able to reduce lung virus titre. However the severity of disease, as assessed by lung pathology or weight loss is increased. Th1 CD4 T-cells were able to augment disease but in these experiments, IL-4 secreting Th2 CD4 T-cells were more potent both at increasing disease and reducing viral load. CD8 T-cells

have been shown both to be more and less potent at augmenting disease than CD4 T-cells

depending on the experimental system (Graham et al., 1991; Alwan et al., 1992; Alwan et a., 1994).

There are conflicting data as to the Th phenotype of cells generated by RSV infection. Analysis of

BAL washes from Balb/c mice infected with RSV showed both Th1 and Th2 T-cells, although the

major producers of IFN-gamma were NK cells, early in infection, and CD8 T-cells later in infection

(Hussell et al., 1996; Srikiatkhachorn & Braciale, 1997b; Hussell & Openshaw, 1998). In humans,

serum levels of Th1 or 2 cytokines are not correlated with disease severity. In vitro stimulation of

PBMC from RSV patiants with RSV antigen gave high levels of IFN-gamma and low levels of IL-4

and 1L-10 (Brandenburg et al., 2000a). Memory responses to RSV have been shown to be

predominately of the Th1 phenotype (Anderson et al., 1994).

Th2 responses to RSV were found when PBMCs were cultured with live RSV but not with UV

inactivated RSV. Several groups have observed that the immune response to G protein is Th2

directed (Jackson & Scott, 1996; Srikiatkhachorn & Braciale, 1997a) . A piece of evidence

suggesting that a Th2 response may not be beneficial is that higher type 2 cytokines levels were

found in the PBMCs of children with RSV bronchiolitis than in healthy donor children. It is

interesting to note that CD8 T-cells were the predominant IL-4 secreting cell in this study (Bendelja

et al., 2000). Also there is a correlation between Th2 cytokines and an early experience of RSV.

Early exposure to RSV is predictive of a poor disease outcome (Roman et al., 1997).

A mouse model was developed to investigate the reasons for the failure of a formalin inactivated

RSV vaccine. This not only failed to protect from disease for a prolonged period, but also caused

enhanced disease in vaccinated children who subsequently were infected with RSV. This disease,

which was fatal in two cases, was characterised by an intense lung inflammatory infiltrate including

eosinophilia (see section 1.5.2) (Chin et al., 1969; Kapikian et al., 1969; Kim et al., 1969). Similar

immunopathology can be induced in Balb/c mice (and other animal models e.g. cotton rats) by

priming with RSV G-protein or formalin inactivated RSV, and later challenging with live RSV

(Openshaw et al., 1992; Woolums et al., 1999; Ponnuraj et al., 2001; Prince et al., 2001). This disease is also characterised by an eosinophil influx to the lung and by the induction ofTh2 phenotype T-cell responses (although high levels of IFN-y from other sources may still be present) (Spender et al., 1998). Blocking of Th2 responses using anti IL-4 and IL-10 monoclonal antibodies or Th2 cell depleting antibody prevents immunopathology (Connors et al., 1994; Walzl et al., 2001). The initial priming event does not induce CD8 cell activity. Hussell and colleagues demonstrated that C57BL/6 mice that do not develop immunopathology when primed with G and later challenged with RSV generated CD8 T-cell responses to G whereas Balb/c mice did not. Moreover when C57BL/6 mice were rendered unable to generate CD8 T-cell responses, by knocking out their O_2 microglobulin gene, they developed immunopathology (Hussell et al., 1997; Hussell et al., 1998). More recently, it has been shown that activated IFN-gamma secreting NK cells are able to protect from vaccine enhanced disease in the Balb/c mouse model (Hussell & Openshaw, 2000).

The relevance of this sensitisation and challenge model to RSV bronchiolitis in naturally infected children is limited as severe disease in humans normally occurs upon first exposure to RSV. It does however provide information as to the role of CD8 cells in regulating other cell types during the clearance of a natural infection.

CD8 T-cells recognise cytosolically derived antigen in the context of MHC class 1 molecules. Because of this ability to detect intracellular infection they are believed to be key cells in the clearance of viral infections. N, SH, M, M2, and NS2 specific CD8 T-cells have been found in the blood of RSV exposed humans (Cherrie et al., 1992). In children these cells appear within 10 days of infection and increase in number throughout the infection peaking at the time of disease resolution (Collins, 2001). Passive transfer experiments in mouse models of RSV infection have demonstrated the ability of CD8 cells to clear virus, but also implicated them in immunopathogenesis (see above). The presence of CTL (which were not definitively characterised

as CD8 T-cells) was associated with decreased severity of clinical disease in humans (Isaacs et al., 1987; Domachowske & Rosenberg, 1999; Rosenberg & Domachowske, 2001). However others have suggested that rapid development of CTL responses leads to wheezing and/or bronchiolitis, whereas gradual development of this response leads to less severe illness. In both humans and mice, the presence of RSV specific CD8 cells after the resolution of disease has been shown to be predictive of later wheezing (Openshaw et al., 2001).

Collectively, these data indicate that both Th1 and Th2 CD4 T-cells are induced by RSV infection of humans, although the predominant cytokine in the lung is IFN-y secreted by CD8 T-cells, monocytes and probably NK cells. Which Th phenotype, if any, is best at preventing severe disease is unclear, although the balance of evidence points towards Th1. The CD8 T-cell response is concomitant with virus clearance, and is also extremely important in avoiding CD4 T-cell mediated immunopathology upon re-infection. CD8 T-cell activation has both beneficial effects but may also cause immunopathology when generated in excess.

1.16 Humoral response

Infection with RSV elicits an antibody response to all of the viral structural proteins. The antibodies directed towards the F and G proteins are the most important for protection (Hall et al., 1991). There are a number of strands of evidence that show that antibody has the ability to protect from RSV infection. There is a brief period of 5 to 6 weeks after birth when maternal antibody titres are highest and RSV infection is rare. Also the levels of antibody in cord blood have been shown to be inversely correlated with severity of subsequent RSV disease (Holberg et al., 1991). There is an inverse correlation between virus neutralising antibody level in the respiratory tract and ability to experimentally infect animals or adult humans (Mills et al, 1971; Hall et al., 1991; Grahamet al., 1993).

Hypotheses to explain the relative susceptibility of young infants to RSV is that titres of anti F and G glycoprotein antibodies (both serum and sIgA) induced in young infants are significantly less than that in older individuals. The limited quantity of antibody that infants make shows little neutralising activity (McIntosh et al., 1978; Murphy et al., 1986).

The formalin-inactivated vaccine that was tested in the late 1960's induced an immunopathological response in some children upon subsequent infection with live RSV. This induced antibody response, was directed mainly at the F protein, and with low neutralizing activity. Until recently this aberrant antibody response had been hypothesized to induce immunopathology. Better understanding of the cellular immune response has implicated unbalanced T-cell responses in the vaccine-induced disease. With the possible exception that high serum IgE levels after infection and severe disease are correlated, (more probably a by-product of a harmful Th2 response, rather than evidence for IgE mediated immunopathology) there is little evidence of antibody driven immunopathology. Given the current view of the protective and benign effects of antibody in RSV infection, administration of RSV-F specific monoclonal antibody is now successfully used as an immuno-prophylactic in high-risk infants (Groothuis et al., 1995; Groothuis et al., 1998). This also has a marginally beneficial effect when given to already infected infants (Malley et al., 1998). Although anti-viral antibody levels may mediate protection from disease they are not necessary for successful recovery from disease as evidenced by the clearance of virus from hypogammaglobulinaemic and agammaglobulinaemic patients. Moreover, Graham et al. (1991) demonstrated that antibody was not necessary for the clearance of RSV infection in mice. Repeated infection with RSV throughout the duration of life is the norm, and although antibody has some protective effect, re-infection can occur in the presence of relatively high levels of RSV-specific immunoglobulins.

Vaccination

There is no vaccine currently available for RSV, but given the high rate of re-infection with RSV it is unlikely that any vaccine will induce life-long sterile immunity.

The minimum requirements for a successful vaccine in infants would be to protect the child from lower respiratory tract infection for it's first or possibly two RSV seasons. This is the period when infection is most dangerous. This essentially is the role of the current prophylactic treatment, passive transfer of RSV specific antibody, but a vaccine would be cheaper and hence more widely available. A successful RSV vaccine for the elderly needs to be at least analogous to the current once yearly influenza vaccine, providing protection for at least one RSV season.

1.17 Live attenuated vaccines

A live attenuated vaccine must strike a balance between immunogenicity and pathogenicity. Over successive decades this balance has proved difficult to achieve for RSV. When tested in adult volunteers or infants trial vaccines have been found to be either over-attenuated and showing no protective effect, under-attenuated and demonstrating a pathogenic effect or genetically unstable and showing a propensity to revert to wild type (Kim et al., 1973 ; Belshe et al., 1982; Wright et al., 1982).

In the last ten years Murphy and his co-workers have developed a new generation of genetically defined live attenuated vaccine candidates (Crowe, 1995; Whitehead et al., 1999; Wright et al., 2000). The viruses were attenuated using a combination of cold passaging, selecting for temperature sensitive mutants (cpts), and chemical mutagenesis. The new viruses were then genetically sequenced both to discover the mechanism of attenuation and to allow identification of reversion mutants. One of these candidates provided protection in chimpanzees, even when they were infused with anti-RSV antibody prior to immunisation. This suggests that this vaccination may be able to overcome the problem of maternal immunity. A development of this candidate vaccine, called; cpts-248/404 went through phase 1 clinical trials and was found to be genetically stable and

immunogenic. However in very young infants the virus was found to be pathogenic (Wright et al., 2000).

Other approaches have been to delete whole genes from the RSV genome. A NS1 and M2-2 negative mutant has been shown to be immunogenic and safe in a chimpanzee model. Human trials have not yet been attempted (Teng et al., 2000).

Given that RSV itself does not induce lasting protective immunity this would not seem a logical route to take. If it was shown that the reduced severity of RSV re-infection was due to immune mechanisms as opposed to age related factors, protection from severe disease may be possible.

1.18 Inactivated RSV vaccines

An RSV inactivated vaccine, consisting of formalin-inactivated virus in alum, was developed. In clinical trails it produced neutralising and complement fixing antibodies in serum. Despite this, the number of vaccine recipients who developed lower respiratory tract complications upon subsequent RSV infection was greatly increased, with 80% of the vaccinees requiring hospitalisation. Sadly, two infants died. Post-mortem examination of these infants and studies of the other vaccine recipients showed a large mononuclear cell and eosinophil infiltrate in the lungs suggesting an immunopathological cause for the disease (Chin et al., 1969; Kapikian et al., 1969; Kim et al., 1969). Inactivated vaccines induced antibody responses that were different from those after natural infection. Formalin inactivation also adversely affected the antigenicity of RSV F-protein. Inactivated RSV also failed to induce anti-G protein antibody. RSV antigen was shown to induce an exaggerated proliferative response in vaccinated children (Openshaw et al., 2001).

Hypotheses proposed to account for vaccine-enhanced disease initially centered on differences in antibody production between inactivated vaccine and natural infection. However in the RSV system, it has become increasingly clear that the T-lymphocyte response induced by inactivated vaccine is inappropriate. The formalin-inactivated virus in alum is unable to produce IFN-y

producing CD8 T-cells (Graham et al. 1993), which are necessary to prevent immunopathology in mice (Tang & Graham, 1997). The current view is that protection from RSV requires a balanced immune response and that domination of one T cell phenotype over the others leads to immunopathology (Openshaw et al., 2001).

1.19 Subunit vaccines based on RSV proteins

RSV vaccines using purified or recombinant viral proteins have also been tested. Immunisation with the F and/or G proteins has been shown to be induce antibody and T-cell responses that reduce the level of virus replication in lungs of mice (Trudel et al., 1989; Tebbey et al., 2000) and cotton rats (Walsh et al., 1987). A number of studies have linked the reduction in lung virus titre with enhanced lung immunopathology, probably because of the paucity of CTL and Th1 responses generated by these non-replicating immunogens (Connors et al., 1992a). Successful clinical trails immunising with purified fusion protein (PFP-2) in alum have been carried out in seropositive children with RSV predisposing conditions (eg. bronchopulmonary dysplasia) and in adults. As these individuals have already been exposed to RSV, the possibility of priming for vaccine-enhanced disease is removed, making this a suitable vaccine candidate in these populations.

Replicating recombinant vaccina virus expressing RSV F protein and/or G protein have been constructed. These provide protection from disease (largely mediated by antibody) without priming for immunopathology in rodent models. A vaccinia M2 protein construct also protects from disease but its action is mediated by CD8 CTLs (Connors et al., 1992a; Connors et al., 1992b; Wyatt et al., 1999).

1.20 Vaccines based on the G gene

There have been several vaccines based on the G gene because of its importance in provoking

immune responses. There is a vaccine, BBG2Na, in Phase 1 and 2 clinical trials that incorporates the conserved region of the RSV G protein and the first variable and second variable regions joined to albumin binding region of streptococcal protein G to increase its half-life when expressed in *E. Coli*. The fusion of the two proteins produced a strong antibody response in mice (Libon et al., 1999). The adsorption to alum makes the vaccine candidate BBG2Na more immunogenic and has proved promising in animal models (Power et al., 1997). When tested in neonatal mice, adequate protection was provided, showing that the vaccine is efficient in the presence of maternal antibody and when the immune responses are immature. Also, the vaccine managed to induce balanced Th1 and Th2 responses (Siegrist et al., 1999). A further study in 2000 showed that BBG2Na increases protective efficacy of serum antibodies in RSV seropositive mice. These results show that the vaccine could be used in the seropositive population providing protection from subsequent RSV infections (Goestch et al., 2000). Two different plasmid vectors encoding cytoplasmic or secreted forms of BBG2Na when immunized in mice produced protective immune responses (Andersson et al., 2000).

Another vaccine candidate was produced from a cDNA clone containing all the genes of the A2 long strain of RSV plus the G gene of RSV B inserted at the A2 F/M2 intergenic regions (Jin et al., 1998). The addition of plasmids encoding the N, P and L genes were needed to recover infectious virus, which showed stable expression of chimeric DNA. Another study showed that efficient recovery of cloned virus needed the M2-1 protein as a separate plasmid (Collins et al., 1999a). This chimeric vaccine expressing the G protein of RSV A and B may prove to be useful.

Anti-peptide antibodies have been shown to neutralize RSV infection *in vitro* and reduce lung titers and lung pathology. The short central conserved region in the G gene may be used for designing a peptide antibody response, which has cross reactivity between types (Walsh et al., 1998).

Vaccination with plasmid vectors encoding full length/secreted G protein (DNAG) elicits balanced

systemic and pulmonary Th1 and Th2 cytokine responses in mice and not atypical pulmonary inflammatory reaction post RSV challenge in cotton rats. Therefore pulmonary Th2 cytokine responses and enhanced lung pathology are not intrinsic properties of RSV G protein. DNAG conferred evident neutralizing antibody responses and protection in mice and cotton rats (Li et al., 2000).

The lack of enhanced lung disease may relate to the animal model. The G protein has not been reported to induce eosinophilia in cotton rats therefore the mouse model may not be as good the cotton rat model.

Plasmid DNA vectors deliver RSV G in the absence of other antigen /adjuvant. Also, they induce balanced Th1/Th2 responses, regardless of the route of administration same as seen as with intranasal infection. In contrast immunization with formalin inactivated RSV for G protein always elicited Th2 response.

Increased production of IL-5 and increased lung pathology is more specifically linked to response to secreted form of G protein in mice. Priming with secreted glycoprotein G of RSV antigens enhances IL-5 production and tissue eosinophilia after RSV challenge. Therefore, RSV secreted G may be important in evading the host immune system and a determinant of disease pathogenesis (Johnson et al., 1998). The amino acids 193-203 are responsible for enhanced pathology including pulmonary eosinophilia in mice (Sparer et al., 1998).

Experimental systems, however, might cause problems such as the effect of adjuvant and/or carrier. Recombinant vaccinia viruses may modulate the immune response to the G protein because the proteins expressed are involved directly in evasion of the host immune defence and may alter the immune response to other antigens (Smith et al., 1997).

However, the G protein has also been shown to cause immunopathological effects producing pulmonary eosinophilia upon subsequent challenge (Hancock et al., 1996).

The variation seen within the G gene suggests there is an immune or selective pressure on this region. Therefore any vaccine produced needs to create the correct immune response, to protect against re-infection and to prevent enhanced illness upon natural infection.

1.21 Treatment and control

There are no effective antivirals used in RSV infection on a routine basis. However, immunoglobulin is often given to prevent RSV infection in certain circumstances.

RSV-IG is produced by screening plasma donors for high titres of RSV neutralizing antibodies and purification follows. This treatment commonly known as Respigram is licensed in the United States. A blind, randomized trial conducted by the PREVENT group on premature infants with and without bronchopulmonary dysplasia showed a reduction in hospitalizations as a result of RSV associated illness, and a decrease in time spent in hospital and decrease in symptoms (Smith et al., 1997). Another trial involving infants and children with congenital cardiac disease showed that RSV disease was prevented in infants less than six months old (Groothuis et al., 1993). However, after the trial, children with cyanotic congenital disease were recommended not to receive RSV-IG. Disadvantages of the treatment include intravenous doses administered monthly during the RSV season and treatment is expensive. Also, it has been shown that once lower respiratory symptoms of RSV infection have started the passive administration of RSV IG had no beneficial effects.

Monoclonal antibodies are also used to treat and prevent RSV infection. Antibodies to the fusion protein are often used because it is highly conserved between subtypes. It is possible that the monoclonal antibody will be administered intramuscularly or perhaps nasally. Trials are being performed to assess efficacy (Weltzin et al., 1996). The main advantages of this type of treatment is that it is less expensive and also there is more viral neutralizing ability per gram, which may reduce

the volume needed for protection.

Humanized mouse antibodies are also being evaluated at present. A study performed by Meissner in 1999 used an antibody against the F protein, but the results were not favorable as no differences between placebo and treated were observed (Meissner et al., 1999). Another humanized antibody (MEDI-493) palivizumab was successful in clinical trials, shown by a reduction in hospitalizations (Subramanian et al., 1998). It has now been licensed and is used as prevention for RSV infections.

Ribavirin is a broad-spectrum antiviral and has been used in treatment of measles, parainfluenza and hepatitis viruses. Ribavirin has many toxic qualities including the reduction of red blood cells (Furne et al., 2003). The Food and Drug Administration (FDA) has licensed aerosolised ribavirin for treating infants with RSV, but it has not been licensed for adults. It probably is not worth using as an antiviral because it is very expensive, difficult to administer and only slightly decreases viral load. However, ribavirin is known to decrease the length of hospitalization for patients with severe RSV related LRTI (Levin, 1994).

Probably one of the most effective methods of preventing RSV transmission is isolating infected individuals. In order to reduce infections there should be avoidance of infected adults, and frequent hand washing. Day-care facilities are also often associated with RSV infections presumably because of potential for viral transfer and spread. Improved patient management results in fewer nosocomial infections by early identification of RSV infection (Karanfil et al., 1999).

1.22 Aims of this project:

1. There is little available published information concerning the molecular   epidemiology of RSV in the Middle East, especially the Gulf region. Accordingly, it was proposed:

a) to document the incidence of RSV infection in Qatar in children younger than 2 years of age; and

b) to use retrospective data to determine the effect of temperature, rainfall, humidity etc. on the incidence of RSV infection over a five year period (1994-1998). The results of this work are given in chapter 3.

2. It has been hypothesised that the C-terminal region of the RSV G protein includes epitopes important for virus neutralisation and that loss or masking of the terminal amino acids can allow the virus to escape from antibody neutralisation. It was therefore proposed to investigate strain diversity of RSV G protein (especially RSV B) by:

(a) analysing the distribution of RSV strains of A and B subtypes by Multiplex PCR of the N/P gene;

(b) performing sequence analysis of the complete RSV G gene from representative isolates as well as clinical material from Qatar; and

(c) comparing sequences from Qatar isolates with those from elsewhere. The results of this work are presented in Chapter 4.

3. Finally, it was proposed to investigate the immunological responses to RSV B strain by:

(a) synthesising overlapping peptides of the C-terminal region of the RSV B G protein and to perform epitope mapping with these peptides;

(b) evaluating the reactivity to these peptides in acute, convalescent and sequential sera from the same individuals; and

(c) determining any correlation between RSV neutralising antibody titre with anti-peptide reactivity in the same samples. The results of this work are given in Chapters 5 and 6.

2   Chapter 2: Materials and Methods

Variation in RSV detection with season of the year

2.1   Setting and Medical Care

2.1.1   QATAR

The state of Qatar lies half way along the western coast of the Arabian Gulf, with an area of 11,437

sq. km. It has a moderate desert climate with long hot summer and short winter with little rain. The

native population is about 500,000, most of whom live in the capital city (Doha), males

predominate over females and the number of foreign expatriates exceeds the number Qatari

nationals, most of whom come from the Indian subcontinent (HMC, 1994).

2.1.2   HMC

Hamad Medical Corporation (HMC) comprises three major hospitals, which are the main hospitals

in the state of Qatar with a total capacity of 1292 beds. They are; Hamad General Hospital (660

beds) encompassing the Departments of Medicine, Surgery, Paediatrics, in addition to specialised

intensive care units and Cardiology and Dialysis units; the Women's Hospital (288 beds) with

Obstetrics and Gynaecology and a Neonatal Care unit; and Rumailah Hospital, which provides care

to the elderly and disabled people in Qatar. Thus all paediatric hospital attendance occurs at one

hospital (HMC, 1994).

2.1.3   Meteorological data collection

The weather and climate data are recorded at Doha International Airport. The observatory is

operated and maintained by the Department of Civil Aviation and Meteorology of the Ministry of

Communication and Transport, State of Qatar (Long period Means and Extremes of

Climatologically Element 1962-1998). The mean values of monthly temperature, evaporation,

relative humidity, total rainfall and wind speed were calculated from the data base for the study years (1994 – 1998).

2.2   Study population and data collection

The study population was all children presenting or admitted to HMC between November 1999 and April 2000 (a six-month period) and meeting the following population case definition:

Lower respiratory infection (LRI) or acute bronchiolitis, presenting within five days of onset of the illness (Wright, 1996).

Data analysis used Microsoft Excel. The data on admission, discharge and RSV incidence were obtained from Health Information System (HIS) department at HMC.

Parents and Ethical Committee agreement had been obtained before sample collection for this Qatar study project (**Appendix 1**).

2.3   Sample collection

Nasopharyngeal aspirates (NPA) were obtained from children presenting to HMC and meeting case definition on each working day during the study period. Wherever possible additional respiratory samples were collected from children found to be RSV Ag positive. Paired sera were also collected from previously healthy children in accordance with the Study protocol. NPA and serum samples were stored at -70°C until required.

2.4   EIA (RSV Abbott Test Pack – Screening test)

A method for the detection of respiratory syncytial virus that utilizes a solid phase enzyme immunoassay to detect viral antigen present in nasopharyngeal aspirates, washes and swabs was used. This was performed in accordance with the manufactures instructions for all samples received from the serology laboratory at HMC and for all NPA samples.

2.5   PCR (Multiplex PCR – Confirmatory test)

A nested Multiplex Rt-PCR containing primers for detection of Influenza A (H1N1, H3N2), Influenza B and RSV A and B (nucleor protein), which has been developed for detection of these viruses in respiratory samples at CPHL, was used to analyse the samples assayed positive by the ABBOTT Test Pack and also to assay negative samples (Ellis et al., 1997).

All PCR work was carried out in conditions designed to prevent the occurrence of carry-over contamination. Nucleic acid extraction, PCR reaction mixes set-up, thermo cycling and gel analyses were all conducted in separate rooms. Transfer of primary product to the secondary reaction mix was done at a PCR workstation, which was equipped with UV light. Each room had dedicated equipment and clothing, and all pipette tips used were plugged to prevent any possibility of contamination. All work was carried forwards from RNA extraction through gel analysis of the final PCR amplicon. Each run consisted of 24 samples with negative controls after the sixth sample. Runs were validated when all the negative controls were negative and all the positive controls were positive, with products of the correct size.

Nucleic acid extraction

RNA was extracted from a 100µl volume of each sample (clinical specimens) by the guanidinium thiocyanate-silica binding method (Boom et al., 1990). The clinical specimen was added to a tube containing 835 µl of lysis buffer (120g guanidinium thiocyanate, 100ml 0.1 M Tris-HCI, pH6.4, 22 ml 0.2 M EDTA pH 8.0, 2.6 g Triton X-100) and 15 µl of silica suspension, mixed, incubated for 10 min at room temperature. The RNA bound to the silica was washed twice with 1 ml buffer L 2 (120g GuSCN, 100 ml 0.1 M Tris-HCI pH6.4), twice with 1 ml of 70% (vol./vol.) ethanol, once with 1 ml acetone and then dried at 56°C for 10 min. RNA was then eluted with 30 µl of RNAse free water and 1µl of RNasin enzyme and converted into cDNA by RT-PCR. For reverse transcription, 22.2 µl of RNA were added to a reaction mixture (17.8 µl) containing 20 mM Tris-HCI (pH 8.4), 50 mM KCI, 7.5 mM $MgCl_2$, 1.5mM of each dNTP, 25 ng of random primer pdN6

(Pharmacia), 1.6 μl of RNasin (Promega) and 200 U Moloney murine leukemia virus reverse transcriptase (Gibco BRL). The reaction was incubated at room temperature for 10 min, 37°C for 45 min, 95°C for 5 min and quenched on ice (Boom, 1990; Gibson, 1993).

The primary primers used were (AH1 A and AHI FII for **FLU H1**, AH3 A and AH3 DII for **FLU H3**, BHA A and BHA DII for **FLU B**, RSV 548 and RSV 1366 for **RSV A and B**) (Stockton et al., 1998).

The secondary primers used were (AH1 B and AHI EII for **FLU H1**, AH3 B and AH3 CII for **FLU H3**, BHA B and BHA CII for **FLU B**, RSV 783 and RSV 1117 for **RSV A** and RSV 783 and RSV 966 for **RSV B** (Stockton et al., 1998). Each primer pair was used at 5pmol in the primary amplification and 2.5 pmol in the secondary amplification reaction. For the primary PCR, 20 μl of cDNA was added to 80 μl of reaction mix containing 10-mM Tris-HCI pH8.8, 3.5 mM $MgCl_2$, and 2.5mM KCI and 1.5 μl Taq polymerase. Amplification, using a DNA Engine thermocycler (T3 from Biometra), consisted of 1 cycle at 94 °C for 2 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1min, and 72°C for 1 min. Two μl of primary product were transferred into 48 ul of secondary amplification mix (as above with 0.2 mM each dNTP) .The samples were then incubated for one cycle 94°C for 2 min, then 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min.

Agarose gel electrophoresis

For multiplex PCR amplicon analysis, 15 μl of PCR product were mixed with 2 μl Orange G loading buffer (0.25% Orange G + [10% Ficoll in TE (10 mM Tris, pH 7.4, 1 mM EDTA]) and run on a 1.2 % agarose gel (S B Fine agarose gel, from Seven Biotech LTD) in 1x TBE (89-mM boric acid, 2 mM EDTA, pH8.4). The amplicons were visualized using ethidium bromide staining and photographed using a Polaroid camera.

G gene sequencing

2.6   RSV G primer designs

Primers were designed both by eye and using OLIGO 6-primer design software. The sources of

sequences which were used to design these primers were those deposited in GENEBANK (RSV A2

strain, isolated 1968, assigned accession number AF035006), (Whitehead et al., 1998), and also

described in the following publications: (Wertz et al., 1985); (Cane & Pringle, 1991)for RSV A and

(Roca, 2001 ; Taylor, 2001) for RSV B. Primers were designed to amplify and sequence the

complete G gene for both RSV strains Aand B. Different sets of primers were designed to be as

similar as possible in their physical and chemical characteristics. They were around 20 bases in

length, with a 50% G+C content and annealing temperature 45°C - 60°C.

2.7   RSV G PCR
At the beginning, nested amplification was performed with concentrations of 5 pmol of primers in

the primary reaction and 2.5 pmol in the secondary round reactions. In the primary PCR, 20 μl of

serial dilution cDNA of Qatar/RSVA/2000/10 were amplified in a 100 μl final volume containing

PCR buffer (20 mM Tris-HCI pH 8.4, 50 mM KCI, 1.5 mM $MgCl_2$) 0.2 mM each dNTP and 1.5 μl

Taq. For the second round reaction, 2 μl of the primary product were transferred into 48 μl of the

above mix with 0.3 μl of Taq. Cycling conditions consisted of 2 min at 94°C, then 35 cycles of

94°C for 1 min, 50°C for 1 min, 72°C for 1 min, for both rounds of amplification.

2.8   Optimization of the RSV G PCR

2.8.1   Biochemistry optimization

A/Q/10/00 which gave a strong band at dilution of (1/100) was used to test 12 different Stratagen

Optiprime buffers (see Appendix 2 for composition of buffer).

2.8.2 Annealing temperature optimization

Starting from the best buffer result, the annealing temperature (Ta) for primer length around 20 bases was calculated by using the following equation

$$Ta= 4(G+C) + 2(A+T) \text{ (Dieffenbach, 1993)}$$

2.9 Gel purification

PCR products were excised from the agarose gel using a clean scalpel and purified from the agarose using QIAquick gel extraction columns (QIAGEN) according to the manufacturers instructions and stored at -20°C.

2.10 Sequencing

RSV A and B PCR amplicons were sequenced using the secondary primers with 3.2 pmol concentrations. Sequencing was performed using dye deoxy terminator chemistry and cycle sequencing (Perkin Elmer) according to the manufacture recommendations Samples were run on an Applied Biosystems 373 A sequencer (ABI sequencer). The sequencing gel was 40% Acrylamide gel (25 g of urea (from Sigma), 1g of Resin AG 501-x8 (D) (from Bio RAD) and 7.5 of acrylamide (fromBio-Rad laboratories) made up to 45 ml with distilled water, which was then filtered by using 50mm filter unit (from Nalgene), finally 250 μl of 10% ammonium persulphate (ASP from Amresco) and 22.5 μl of TEMED (from Sigma) were added to the gel solution. Gently and evenly the gel was squirted along edges of ABI sequencer avoiding air bubbles. Sequencer plates (after proper cleaning by water, dionised water and 100% ethanol to dry) were fixed by clips (heating block was adjusted to 95 °C). After 2 hours the clips were removed and 1xTBE (Tris Boric acid EDTA) buffer were added to the machine. To prepare samples for loading, each sample (including PCR sequencer controls) was mixed with 5:1 deionized formamide: EDTA (125 μl deionized formamide to 25 μl EDTA 50mM/De tran Blue 50mg/ml) and incubated for 2 minutes at 95 °C,

then placed on ice for one minute, then spun briefly before loading into the sequencer. The type of run was a full scan that took 14 hours.

2.11   Sequence analysis

Output files from the 373 A sequencer were imported into SeqED (Applied Biosystems) for comparison of forward and reverse sequence.

Unreliable data were removed from the beginning and end of the sequence. Edited files were transferred to the EditSeq programme (Lasergene, DNA Star). From this format the sequences were exported into MegAlign (Lasergene, DNAStar) where they were placed in frame and aligned using the Clustal method. The Clustal method groups sequences into clusters by examining sequences between pairs. The clusters are aligned as pairs, then collectively as sequence groups to the overall alignment. After the multiple alignments are completed, a neighbour joining method is employed to construct phylogeny. Sequences were trimmed to be of the same length for each sequence product. Sequences which were aligned in MegAlign (Lasergene, DNAStar) were exported in a Paup format and converted into a Phylip format using the program Readseq. The output files were used to perform the Bootstrap test by two methods,Parsimony and distance for each phylogeny tree in an attempt to see how stable the internal nodes of these trees are by deleting random positions in the alignment and duplicating others to keep the total number of positions the same. The best tree was then recalculated. This was repeated many times (usually > 100) and the number of times a specific cluster was seen was counted. Dr. Graham Clark at the London School of Hygiene and Tropical Medicine kindly assisted with the bootstrap analysis.

2.12   Peptide synthesis

Fifteen overlapping synthetic peptides (1-15) representing amino acid sequence 149-299, of the G protein of RSV (B/Q/28/00). This sequence includes the C-terminal region with two variable

regions separated by the most conserved region in both subtypes A and B and excludes the transmembrane region. The peptides were numbered 1 through 15 starting from the COOH-terminus of the sequence. Peptide 16 represents the most conserved region only and peptide 17 contains the amino acid sequence that has a 4 cysteine noose. Peptides (13, 14, 15, 16, 17) were synthesized by a Pioneer Peptide Synthesis System (Perceptive Bio systems) at LSHTM and the remaining commercially by Peptron Ltd (Seoul, Korea) using same system.

2.12.1 Reagents

All synthesis solvents were of analytical grade. Fmoc-protected amino acids, the polyethylene glycol graft polystyrene (PEG-PS) and HATU were purchased from Novabiochem. Dimethylformamide (DMF) was purchased from Rathburn Chemicals. Piperidine, N, N-diisopropylethylamine (DIPEA) from Fluka and triisopropylsilane were purchased from Fluka. DMF was stored over type 4A molecular sieves (BDH) overnight before use and checking for acidity by mixing a few drops of DMF with Bromophenol blue (200mg/2ml of DMF), which should stay yellow.

2.12.2 Peptide assembly:

Synthesis was performed on polyethylene glycol polystyrene grafts (PEG-PS) resin cartridge. The required amount of resin was first allowed to swell by adding 20ml of DMF and then packed in the cartridge where the peptide was to be assembled. The synthesis proceeds from the carboxyl terminus to the amino terminus of the peptide. Converting it to its ester form with DIPEA/HATU prior to coupling with the N-amino group of the preceding amino acid activates the carboxyl moiety of each incoming amino acid. The temporally protecting group of the N-amino moiety was removed at the beginning of the next synthesis cycle by flushing 20% (v/v) of piperidine in DMF through the column. Excess piperidine was washed out with 12-column volumes of DMF. The machine then

prepared the activated amino acids and introduced them into the sample loop at the correct time. The amino acids were dissolved in the activator solution in sufficient quantity, as calculated by the standard scale program to yield 0.059mmol of amino acid solution. This was achieved by reweighing 0.8mmol amino acids containing HATU to provide the required four-fold excess of amino acids. This generated the maximum possible yield and highest quality of the final product. The washing step was carried out after each step of Fmoc removal or coupling of each amino acid. In addition, reactive side chains on the amino acids were protected with permanent protecting groups. Repeating the synthesis cycle until the last amino acid was assembled extended the peptide chain. At the end of the synthesis, the peptide was assembled in association with the resin. The peptide-resin was transferred to a sintered funnel and then subjected to successive washing steps with Propan-2-ol and scavengers in the presence of KOH, followed by a drying step using a water vacuum. At the end of the synthesis, the side chain-protecting groups were removed.

2.12.3 Peptide-resin cleavage

Peptide was cleaved and side chains deprotected by rocking in 95% Trifluoroacetic acid (TFA from Romil) and 5% scavengers depending upon the amino acid sequence, for approximately 2 hours at room temperature. Water was use as a general scavenger, but for Methionine and Cysteine containing peptides ethanedithiol was used. The resin was then filtered, washed well with cleaving solution and rotator evaporated to oily residues. The peptides were precipitated by the addition of cold diethyl ether (30-40 ml). After 3 washing times with diethyl ether, the peptide was dried and the white powder was collected and stored at -20°C for further use. Peptides for which special chemicals were used as scavengers were dissolved in distilled water/acetic acid and extracted with ether twice, the organic phase was back-washed with water and the aqueous phases, which contained peptide, were collected and lyophilised.

2.13 Peptide Characterizations:

2.13.1 High performance liquid chromatography (HPLC):

The purity of the peptides was assessed by HPLC to exclude contamination with deletion products and to ensure the presence of a single peak of the major product. Using a Shimadzu system with a perfusion reverse phase column (Poros 4.6mmx 10 cm: PerSeptive Biosystems). The column was previously washed with 100% HPLC grade methanol (Rathburns, Scotland) then equilibrated with initial buffer: 5% buffer B in buffer A. [Buffer A = 0.1% TFA/HPLC grade H_2O/ acetic acid. 100 µl of this was injected on to the HPLC column and eluted with a 5- 70% B gradient over 15 minutes at a flow rate of 9 ml/minute detected by UV 214 nm. I thank Dr Patrick Corran at LSHTM for help in making these purifications.

2.13.2 Mass spectrometry:

The molecular weights of all synthetic peptides were determined by mass spectrometry performed by Peptron Ltd (Seoul, Korea) using an HP 1100 series LC/MSD spectrometer.

2.14 Optimization of solid-Phase ELISA

Two human serum samples (one from a recent RSV infection (confirmed by PCR)) with high RSV antibody titres and peptide 11 from RSV F protein, which was highly reactive with human serum, were used to standardize the ELISA technique.

Four different plates (IMMULON 1, IMMULON 2, IMMULON 4 and NUNC) were coated overnight at 4°C with 50µl per well of a 5µg/ml solution of peptide in 0.1 M carbonate- bicarbonate buffer (pH 9.6). The plates were blocked with 4 different blocking reagents (1% gelatin, 5% milk solution, 1% bovine serum albumin/phosphate buffered saline (BSA-PBS) and 1% fetal calf serum (FCS)). Serial twofold dilutions of serum in 0.05% Tween 20 of corresponding blocking solution (final volume 50µl) were added to the plates, which were incubated at 37 °C for 1 hr and then

washed. Fifty microliters of a 1/1000 dilution of peroxidase –conjugated goat anti- human IgG

(DAKO) was added to each well and the plates were incubated for 1 hr at 37 °C. Unbound

conjugate was removed by washing and 50µl of 0.04% *o*-phenylenediamine-hydrogen peroxidase in

citrate/phosphate buffer was added to detect bound enzyme. The reaction was stopped after 10

minutes by the addition of 25 µl of 2 M sulphuric acid per well and absorbance (A_{492}) was

determined in an automatic plate reader (Dynex MRX).

2.15 Peptide screening assay

Different samples were used to test peptide reactivity. Qatar study sera collected before and after

the infection during 2 seasons (35 samples), neonatal sera (mainly maternal antibodies, 20 samples)

and adult serum (6 samples).

IMMULON4 plates were coated with peptides (1-17) [5µg/ml in 0.1M carbonate/bicarbonate buffer

pH 9.6] 50 µl per well and incubated overnight at 4°C. The following day, the plates were blocked

with 1% BSA-PBS solution, serial twofold dilutions of serum in 0.05% Tween 20 in blocking

solution (final volume 50µl) were added to the plates, which were incubated at 37 °C for 1 hr and

then washed. The rest of work was as described in the above section.

2.16 Inhibition assay

Further investigations were carried out using peptide 14 that was recognized by the majority of sera

to find out if this part of the RSV B G protein was bound with high affinity by these sera, which

might suggest that it is immunogenic.

Sera from Qatar children (Q20, 28 and 37) before and after infection with RSV were used. Sera

from all these children showed increasing levels of neutralizing antibodies after infection.

The experiment discussed here was designed to determine if there was an increase in affinity of

binding to peptide 14 (affinity maturation) following an infection. The inhibition assay (Rath et al.,

1988) was used. The inhibition of binding to RSV Ag in the presence of peptide 14 was measured. Peptide 9 (non-reactive peptide) was used as a negative control and RSV antigen as a positive control (RSV Ag is a long strain of RSV grown on a monolayer of MA (Meth A) cells) from Biogenesis). The free peptide concentration of peptides 14 and 9 as well as the concentration of RSV Ag and the serum dilution that would be used in this assay were previously determined for this experiment.

The serum dilutions to be used had been determined using RSV Ag (at 3µg/ml)- coated plates at the dilution that gave an OD reading of 0.7 (i.e. dilutions 1/40 and 1/20 were used for sera Q20 before and after, respectively, dilutions 1/20 and 1/320 were used for sera Q28 before and after respectively and dilutions 1/20 and 1/160 were used for sera Q37 before and after, respectively). Three IMMULON4 plates were coated with RSV Ag 3µg/ml in 0.1M carbonate/bicarbonate buffer pH 9.6, 50 µl per well and incubated overnight at 4°C. The following day, plates were blocked with 1%BSA-PBS solution for 2hours at 37°C, and washed 5 times with tap water. 21 µl of diluent (1%BSA/PBS/0.05% Tween 20) were placed in each well. Three inhibitor concentration were used [10µl of 1mg/ml of peptides 14 and 9 or 6µg/ml of RSV Ag +21 µl of diluent] were added to appropriate 8 wells and were titrated across the plate using half log dilutions. 25 µl of the representative serum dilution were added to appropriate wells. The remaining steps were similar to the Solid Phase ELISA assay described in section 2.15.

From the inhibition assay the log concentration of peptides (14 and 9) and RSV antigen, which required to inhibit 50% of the maximum binding as an index of affinity was obtained.

2.17 Virus isolation

2.17.1   Cells

Hep-2 cells were obtained from the European Tissue Culture collection (ETCC). Cells were grown in growth medium (minimal essential medium (MEM)(Gibco-BRL) supplemented with 10% FCS at

37°C in a 5% CO_2 incubator. Cell counts were performed and cells were seeded at 5×10^4 cells/ml to give 80-90% confluence the next day.

2.17.2 Virus growth

2.17.2.1 Control RSV strains

Four RSV strains were used in the development of RSV enzyme immunoassays, neutralization tests and for PCR controls (RSVA long and A2 strains from American Tissue Culture Collection (ATCC), RSVB VS 907 and RSVB VS 1039 95-9085. (Kindly supplied by Dr. Pat Cane of Birmingham University) and RSV B N2 (from ATCC). Prior to infection with RSV, 5×10^4 Hep-2 cells were grown overnight to 80% confluence and medium removed prior to inoculation. Virus containing medium was gently dispersed over the whole cell sheet and the culture was incubated at 37°C for 2 hours. Every 30 minutes, the flask was gently rocked to disperse the medium and then rotated in the incubator for even distribution of virus over the cell sheet. A control flask was set up in the same way with 2 ml of MEM 2% FCS. After 2 hours incubation, excess virus stock was removed from the flask and replaced with 20 ml 2%medium. The flasks were then incubated at 37°C in an atmosphere of 5%CO_2 in air and examined daily for the presence of a typical CPE and the formation of syncytia. When 75% of the cell sheet was affected (2-4 days for RSV A and 3-4 days for RSV B post infection) the contents of the flask were harvested using a cell scraper to remove the cell sheet from the flask. The contents of the flask were then pooled and gently dispersed prior to aliquoting into suitable amounts on ice and stored at -70°C for subsequent use.

2.17.2.2 Field strains Isolated from Qatar samples:

RSV positive NPA samples from Qatar were inoculated onto 80% confluent monolayers of Hep-2 cells and incubated at 37°C, and monitored daily. The infected cells were passaged when the viral CPE showed 60-70% of cells infected. Qatar isolates were named with an initial letter indicating

subtype (either RSV A or B). The next number indicates the year that the sample was collected (99 or 2000), the following number indicates the study sample number (e.g. Qatar/ RSVA/2000/10).

2.17.2.3 Preparation of isolate for electron microscopy:

The isolate was grown as previously described, the supernatant was removed and the cells were covered with 3% phosphate buffered glutaraldehyde (12ml of 25% glutaraldehyde and 88ml of Modified Millonigs Phosphate buffer [18.76g sodium dihydrogen phosphate, 4.28g sodium hydroxide and 1 litre of H_2O]) overnight at room temperature. Mr.Bart Wagner from the Northern General Hospital performed the electron microscopy.

2.18   Virus titration

A checkerboard design was used to determine the optimal virus dilution of the control strains and isolate of RSV and the optimal primary and secondary antibody dilutions. Hep-2 cells were seeded at 5×10^4 cells/ml in minimal essential medium (MEM) (Gibco-BRL) containing 2% foetal calf serum in 96 well microtitre plates (Greiner Labortechnik) and incubated overnight at 37°C in an atmosphere of 5% CO_2 in air. 100 µl of each virus dilution were added in duplicate to wells containing cell monolayers. The plates were sealed and centrifuged by Sorval Refi Centrifuge at 1,500 rpm for 45 min at 37°C and incubated at 37°C for 1 hour 15 min. Samples were aspirated from the wells and replaced with MEM medium containing 2% foetal calf serum and further incubated for 3 days at 37°C in 5% CO_2. The medium was removed from the plates and the cells washed with cold phosphate buffered saline (PBS) prior to fixation for 20min with absolute methanol contains 2% (vol/vol) hydrogen peroxide. 100 µl of 5% milk solution (Marvell: dried skimme milk) in PBS were added to each well for 30 min.

Virus infected cells were detected by the addition of polyclonal goat anti-RSV antibody (Chemicon Inc. USA) diluted 1:1000 in 5% milk for 1 hour at room temperature. Wells were washed with 5% milk solution in PBS for 30 min.

Rabbit anti-goat horseradish peroxidase (HRP) conjugate (Chemicon Inc. USA) diluted 1: 2000 in 5% milk was added to each plate, incubated for a further hour at 37°C. After washing four times with PBS using a Denley washer, 100μl of 3,3` diaminobenzidine (DAB 10mg/ml), PBS 40 ml and 30% H_2O_2 13.3 ml) substrate was added to each well and incubated at room temperature for 30 minutes. The substrate was removed and wells were washed 2 times with sterile water. Wells were examined under the microscope. Each plaque appeared as a brown cell/syncytia. Infectious foci were counted in each well of each serial dilution. The serial dilution of virus that gave 100 (or the maximum) number of plaque per well was used to calculate the number of infectious foci per ml (pfu) for each strain (Chapter 6, section 6.5 Final RSV micro neutralizing assay).

2.19 Evaluation and standardisation of cell based foci forming unit assay of RSV using DAB

2.19.1 Monoclonal antibodies:

Selection of both commercial and non-commercial monoclonal and polyclonal antibodies was achieved using a cell - based ELISA with DAB substrate as previously described. The best conditions were selected for use in the neutralization assay (described below).

2.19.2 Incubation periods:

Different incubation periods were tested for virus growth (1, 2, 3, 4, 5 or 6 days).

2.19.3 Fixation methods:

Two types of fixation reagent were tested, absolute methanol containing 2% (vol/vol) hydrogen peroxide or absolute methanol containing 50% (vol/vol) acetone.

2.20 Preparation of neutralizing rabbit anti sera

To prepare crude culture supernatant 2 RSV strains (RSV-A (strain long) and RSV-B (strain Vs 1039)) were grown in Hep-2 cells as previously described and then harvested (supernatant and cells) into 20 ml universal plastic tubes. They were centrifuged in a bench centrifuge (MSE centrifuge; 2000 rpm/ 20 minutes) and the supernatant was removed from the pellet. The pellet was resuspended in 2-3 ml of normal saline, centrifuged for 1 hour, finally the supernatant was removed, and the pellet was dried and resuspended in 200µl of saline. The supernatant from the first centrifugation step was transferred into Ultra-Clean centrifuge tubes. The tubes were centrifuged at 28000 rpm at 4°C for 2 hours (BECKMAN ultra centrifuge SW27 rotor). The supernatant was removed and the tubes were inverted for 10 minutes to dry. 100µl of saline was added to the whitish pellet layer and it was sonicated for 30 minutesin a Grant ultrasonic bath. This crude culture supernatant of HRSV-A (long strain) and RSV-B (strain Vs 1039) was used to immunise two rabbits. Blood samples were taken before and at 2 weeks after immunisation.

2.21 Evaluation of different dilutions of serum

Two human serum samples were used to determine the best serum dilutions to use in the neutralization assay (2-fold and 4- fold).

2.22 Micro neutralization test

Neutralization antibodies were determined by a modification of the micro neutralization method of Anderson et al.(1985). RSV A and B virus stock was diluted 10-fold in MEM medium. Twofold dilutions (starting at 1:5) of test sera were incubated with 100 50% tissue culture infective doses of RSV A (A2 andA/Q/10/00) and B N2 strains for 2 hours at 37°C in 5% CO_2 of 96-well plate. The

Hep-2 cell monolyers were fixed after 3 days of incubation and cell based ELISA assays were performed. Plaques (representing syncytia) and cytopathic effects were to a dilution of 10^{-3} with RSV A and up to a dilution 10^{-2} for RSV B. The mean plaque count was 80 for strain A and 40 for strain B. Therefore, the titre of the virus stock was estimated as:

Number of plaques x reciprocal of the dilution x 1/ vol. of inoculum, viz.

RSV A pfu/ml = 80 x 10^3 x 1/0.2 = 4 x 10^5

RSV B pfu/ml = 40 x 10^2 x 1/0.2 = 2 x 10^4

The expression of viral antigen on the Hep-2 cells was detected by the cell-based foci forming unit assay for RSV using DAB substrate as previously described with a primary antibody to a mixture of 3 RSV proteins F, G and P (MAB858-4 from Chemicon) for RSV A strains and RSV G protein (MAB858-2 from Chemicon) for RSV B strains and anti-mouse horseradish proxidase (from Dako).

3 Chapter 3: The seasonality of RSV infection in Qatar

3.1 Introduction:

RSV is a well-recognized cause of acute lower respiratory tract infection (ALRI) in early childhood and accurate estimates of invasive RSV diseases have been obtained in several industrialized countries (Weber et al., 1998b). However, little is known about RSV infection in tropical and developing countries.

RSV spreads via infected respiratory secretions and transmission occurs mainly through direct close contact with infected individuals or contact with surfaces contaminated with respiratory secretions. RSV can survive on clothes for about 30 minutes and on smooth surfaces like countertops and stethoscopes for several hours (Blydt-Hansen et al., 1999). RSV can cause respiratory tract infections of patients in any age. Clinically severe RSV infections are mainly found in the very young, presumably at their first infection. Reinfection with RSV is common with a milder clinical course and the infection usually remains limited to the upper respiratory tract. Frequent reinfections enable RSV to remain highly prevalent in the population. At least 50% of children are infected during their first winter. By two years of age, almost all children will have been infected at least once and over 50% will have been infected twice (Henderson et al., 1979). RSV infection is seasonal in most countries, outbreaks occur most frequently in the cold season in areas with temperate and Mediterranean climates and in the wet season in tropical countries with seasonal rainfall (Weber et al., 1998a; Weber et al, 1998b). RSV infection is also now well documented as a cause of yearly winter epidemic of acute lower respiratory tract diseases (ALRI), including bronchiolitis and pneumonia.

In developing countries there are few population-based estimates of the incidence of RSV disease, where RSV infections may follow a different pattern (Videla et al., 1998). Viral infections have been found to cause a median of 37% of LRI. Among viral etiologies, RSV is the most frequent, causing a median of 18% of LRI. Applying this proportion to the incidence of etiological agents of

LRI, the estimated incidence of RSV-associated LRI is 97 to 180 episodes per 1000 children / year (Wright, 1996).

Generally, the proportion of those infected world-wide that do not develop symptoms is unknown, however, in industrial areas of Britain, studies of urban population have estimated that 25/1000 infants would develop severe RSV bronchitis and require hospital admission and about 3% of these develop respiratory failure and require ventilation (Smyth et al., 1997; Stott & Taylor, 1985). Factors, which may influence the incidence and severity of RSV-associated disease in developing countries, are passive smoking, malnutrition, asthma, HIV infection, and over crowding (Wright, 1996).

Other epidemiological factors that could explain the reported variation in disease severity are environmental factors such as air quality, which may exert an influence on RSV infection by modulation of the infant's capacity to respond to pulmonary disease by compensatory hyperventilation. Parental smoking also increases the risk and severity of RSV infection. It is important to define the role of air humidity or temperature, either indoors or outdoors, of industrial air pollution, as this may contribute to excess cardiovascular deaths among adults or elderly patients, and could affect the capacity of young infants to cope with respiratory infection (Brandenburg et al., 1997).

Asthma is another factor that may relate to RSV infection. Asthma is a lung disease with the following characteristics: airway obstruction that is reversible, spontaneously or with treatment, airway inflammation, and increased bronchial responsiveness to a variety of stimuli (Dawod & Hussain, 1995). Little is known about the link between RSV infection in infancy and subsequent asthma in developing countries (Weinberger, 2003). Two studies from the Gambia (Weber et al., 1998a) and Qatar (Dawod & Hussain, 1995) noted frequent wheezing episodes in children after RSV infection. A possible explanation is the release into the airways of leukotrienes, which usually induce airway obstruction (van Schaik et al., 2000).

The aim of this part of the work was to document the incidence and seasonality of RSV in Qatar among previously healthy children under 2 years of age. The following methods were used in an attempt to achieve the aims:

Collection and analysis of weather statistics;

Comparison of two RSV detection methods (EIA and reverse transcriptase (RT) -PCR) for screening; and

Retrospective analysis of viral detection data;

Prospective analysis of clinical samples from children admitted with a clinical case detection compatible with acute respiratory infection;

Correlation of prospective, retrospective and viral detection data with weather statistics to determine the relationship of some weather parameters to RSV incidence in a 5 year period; and

Setting up a PCR laboratory at HMC, during 2 RSV seasons of the study period, for the identification of RSV subtypes by RT-PCR.

3.2 Results:

3.2.1 Epidemiological and clinical results:

As described in the Materials and Methods, all hospital admissions in Qatar took place through a single hospital. The total number of admissions to the Pediatric department of HMC over the 6 month period, from November 1999 to April 2000 was 10,435. These included emergency and elective admissions. The main causes of hospital admission, compiled from the health information system of the hospital episode statistics and based on primary admission diagnosis are shown in **Figure 3.1.a.** The sex distribution for admissions of all children under 14 years was 64% male. Diseases of the respiratory system accounted for the majority of admissions (53%) in this age group, with bronchitis and pneumonitis as the primary diagnoses. The most common secondary admission diagnosis was bronchiolitis as shown in **Figure 3.1.b**. Enteric infectious disease accounted for only 7% of admissions in this age group.

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Figure 3.1: Distribution of the main (a) & secondary (b) causes of hospitalization among children in the Pediatric Department at HMC with total number 10,435 (November 1999 to April 2000).

7%

18%

17%

53%

5%

■ Enteric infectious & parasitic Diseases.
■ Disease of respiratory system.
■ Diseases of circulatory system.
□ Diseases of genitourinary System.
■ Injury and poisoning
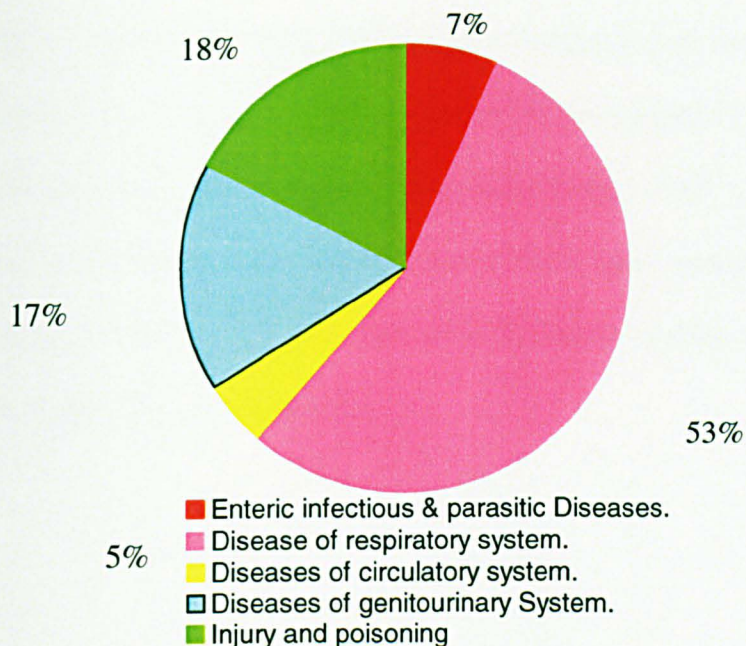
(a) Diseases of the respiratory system were the main reasons for the admission of children.

■ Bronchiolitis ■ Pneumonia ■ Asthma ■ Sepsis ■ Acute tonsillitis ■ Unknown
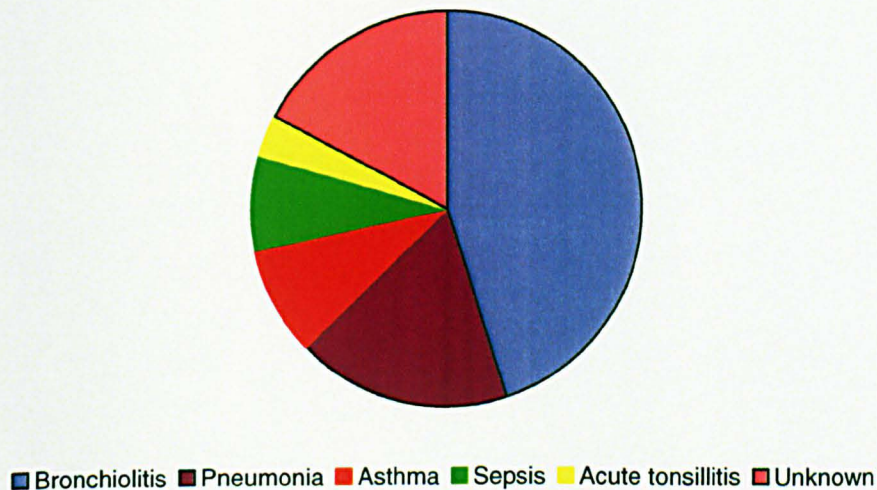
(b) Bronchiolitis was the main respiratory disease causes children admission.

3.2.2 Seasonal distribution of RSV in Qatar

An estimate of the seasonal variation of RSV in Qatar was prepared using retrospective data collected from the HMC hospital microbiology/serology diagnostic laboratory over a 5year period (1994-1998). The respiratory samples analysed by the hospital laboratory were primarily nasopharyngeal aspirates (NPAs) obtained from children of all ages admitted for diseases of the respiratory system. Detection was performed using a rapid antigen detection test (EIA Abbott Test Pack) (Chapter 2 section 2.5). It is clear that there was a marked seasonal distribution of RSV detection (**Table 3.1** and **Figure 3.2**).

Table 3.1(a&b): Seasonal distribution of distribution of RSV detection

Month	Total Number of samples	RSV pos. by EIA (%)
January	595	200 (33.6)
February	316	88 (27.8)
March	185	37 (20)
April	125	19 (15.2)
May	93	12 (12.9)
June	93	17 (18.3)
July	71	14 (19.7)
August	71	18 (25.3)
September	79	40 (30.6)
October	131	40 (30.6)
November	295	159 (53.9)
December	470	221 (47)

(a) Total number of NPA samples and incidence of RSV during the 5 year period (1994 – 1998).

Correlation of RSV/ some weather elements	Coefficient values R	P-values
Monthly mean temperature	- 0.7384	0.006
Mean relative humidity	0.7628	0.0039

(b) Statistical analysis of the correlation between the incidence of RSV and some weather elements by Chi-Sequared test. There is a highly positive correlation between RSV detection and the mean relative humidity with a significant P-value, while there is a negative correlation between RSV detection and the monthly mean temperature with a significant P-value

Figure 3.2: Monthly distribution of RSV positive cases shown with meteorological data over a 5 year period 1994-1998
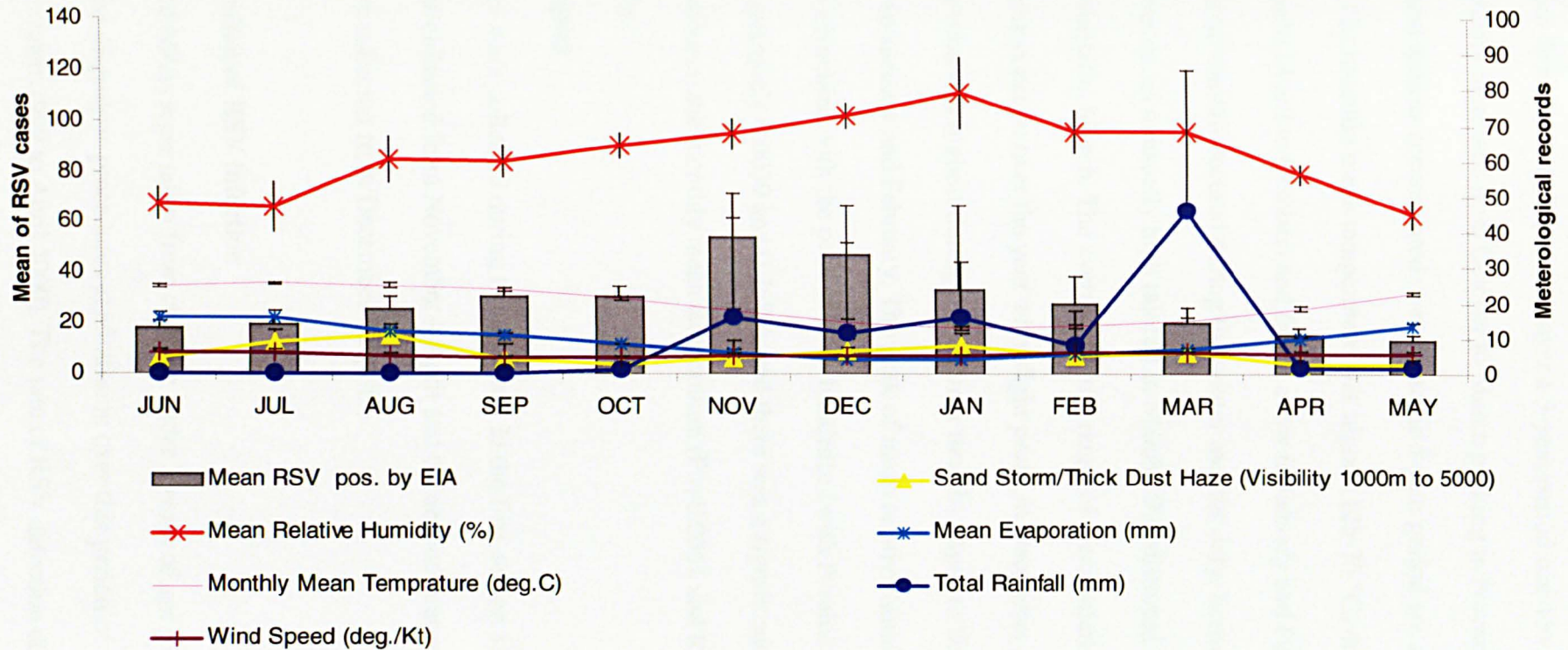
Each point represents mean value (+/- SD). Gray solid columns represent the RSV incidences detected by EIA. Direct relationship between the mean values of RSV detection and the mean of relative humidity (the red line) and the total rainfall (the blue line) values.

RSV detection from April to September over a 5-year period was low. The incidence

of RSV gradually increased from October to March peaking in November.

Meteorological indices accumulated over the same 5-year period are also displayed in

Figure 3.2. The monthly mean temperature was highest (25-35 °C) during the

summer months (April to October) and fell to a low in January and February (17-

20°C). The total rainfall occured during the winter months only, between October and

March. However, an unusually high rainfall in March 1997 distorted the average

figures for rainfall in March. The average annual range of mean relative humidity

remained fairly constant over the year with slight peaks in December and January The

mean evaporation was highest during the summer months (April to September) and

fell to a low in January and February. The peak of mean relative humidity

significantly coincided with the peak of RSV incidence (with P-value and correlation

value (R) respectively 0.0039 and 0.7682), and there was a significant inverse

correlation between the monthly mean temperature (P = 0.0061 and R = - 0.7384) and

RSV infection.

3.2.3 Samples

NPA samples were collected during two seasons. In the first season 1999-2000

samples were collected from November – April and the second season 2000-2001

samples were collected from December – April.

Age distribution of RSV infection

A total of 418 NPAs were taken from children under 2 years of age (58% male) as

part of routine diagnostic procedures at admission over this period of time (first

season: November 1999 to April 2000). The rate of RSV detection decreased with

increasing age of the children sampled (**Figure 3.3**), with 62% of positive RSV

detection in males, reflecting the admission and sampling bias.

3.2.4 Comparison of detection methods for RSV

418 NPA samples were taken from 418 pediatric patients admitted with respiratory

tract infections over the study period. 148 NPA samples (35%) were positive for RSV

by EIA and multiplex PCR and 182 samples (43.5%) were negative by both tests. Of

the remainder, 79 samples were RSV positive by PCR but negative by EIA and 9

samples were positive by EIA but negative by PCR (**Table 3.2**).

Using PCR detection as a gold standard, the sensitivity of the RSV EIA was

calculated to be 74%, and the specificity 95.5%. These data therefore confirm the

validity of using the EIA results to determine seasonality of RSV in Qatar, but

indicate that it will underestimate the total number of RSV cases. A typical gel of the

results of PCR is shown in **Figure 3.4** with subtype A giving an amplicon size of

344 bp and subtype B giving an amplicon size of   138 bp.

3.2.5 Screening samples

A total of 801 NPA samples were collected during two RSV seasons (**Tables**

3.3.a&b). There was no mortality among the RSV positive children during the two

seasons.

Figure 3.3: RSV positive detection (by EIA and PCR) among children of different ages during time for first season. RSV detection significantly decreased with increased age.
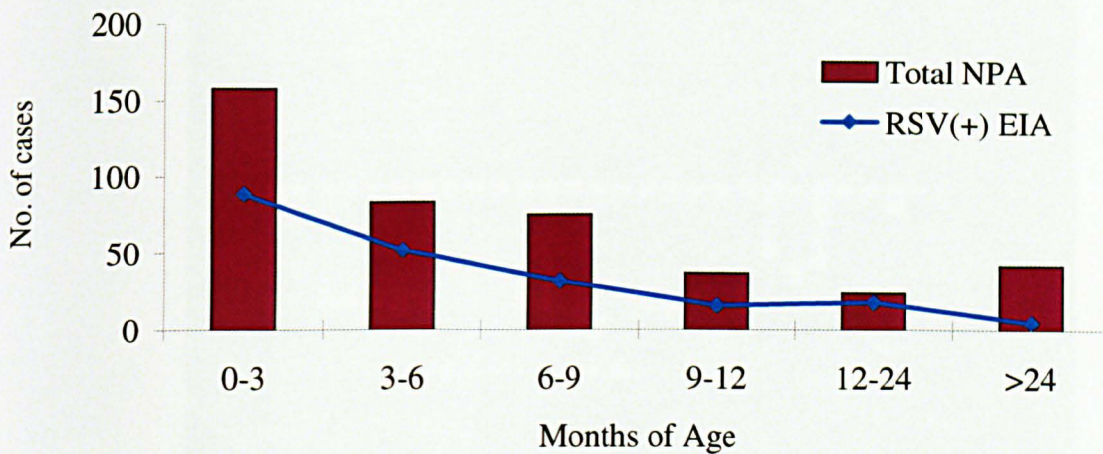
Table 3.2: Comparison of EIA and PCR for RSV detection in clinical samples during first season of the study.

	EIA (+)	EIA (-)	Total
PCR (+)	148	79	227
PCR (-)	9	182	191
Total	157	261	418

Although, 79 samples were positive by RT-PCR and negative by EIA, EIA sensitivity and specificity comparing to the RT-PCR were 74% and 95.5% respectively.

Figure 3.4: Typical gel image of RT-PCR result obtained.
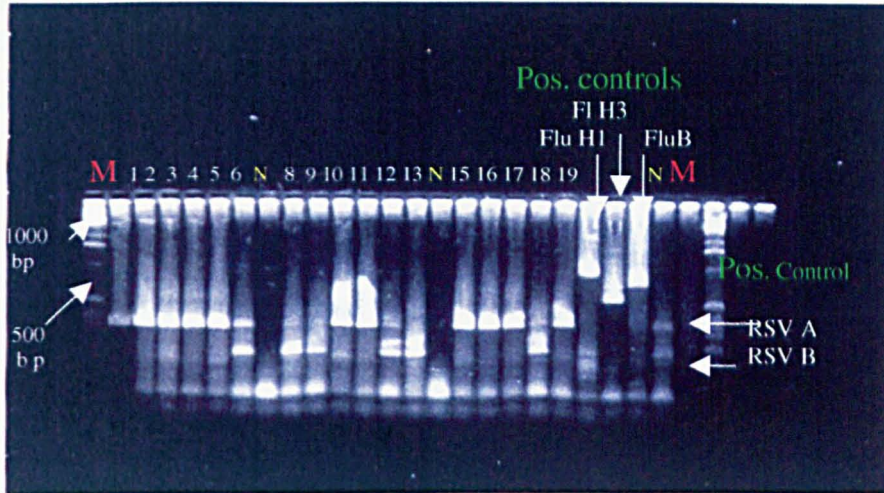
Fragment sizes are RSV A = 344 bp, RSV B=138bp, Flu H1 (influenza A (H1N1)) = 944 bp, Flu H3 (Influenza A (H3N2)) = 591 bp and FLU B (Influenza B) = 767 bp. (N) Negative controls were placed after every 6 specimens and the positive controls at the end of each run. RSV A positive specimens are seen in lane 1,2,3,4,5,10,11,15,16,17 and 19. RSV B positive specimen seen in lane 8,9,12,13 and 18. Dual infection (A&B positive) specimen can be seen in lane 6. M is marker with base pair sizes indicated by arrows.

Table 3.3 (a & b): Relationship between age of child at admission with RSV subtyping.

(a)

Age group (Months)	NPA	RSV PCR or EIA +Ve	RSVA	RSVB	Dual infection RSV A & B or (RSV & Flu)
0-3	158	92	57	24	8
3-6	83	57	31	20	1
6-9	75	35	24	5	3
9-12	37	17	13	0	3 & (1)
12-24	23	19	13	4	1
>24	42	6	4	1	0
Total	418	226 (54%)	142 (67%)	54 (25.5%)	16 (7.5%)

Table 3.4 (a & b): Relationship between age of child at admission with RSV subtyping.

(b)

Age group (Months)	*NPA	RSV EIA+Ve Only	RSVA	RSVB	Dual infection RSV A & B
0-3	112	36	24	11	1
3-6	97	17	7	8	2
6-9	35	6	4	2	-
9-12	26	6	5	1	-
12-24	4	4	3	1	-
>24	9	3	2	1	-
Total	383	78 (20%)	49 (61%)	28 (35%)	3 (4%)

*NPA: Nasopharyngeal aspirates

The total number of NPA samples during study period (first season (Table a) and second season (Table b) 801 was samples and the total positive RSV samples was 304 (38%) with predominance of strain A. There were insufficient NPA samples from remaining 22 samples to allow further investigation.

Analysis of RSV subtype distribution showed that RSV A was the predominant subtype throughout the study. Of the 418 RSV positive samples 142 (67%) and 54(25.5%) were RSV A and RSV B positive, respectively, in the first season and 45(62.5%) and 24(35%) were RSV A and RSV B positive, respectively, in the second season, including some dual infections (16 (7.5%) and 3 (4%) samples for first and second season). The RSV A and B seasons started simultaneously, with the appearance of RSV B detection being higher in the second season.

3.2.6 Qatar study samples

A total of 60 children were eligible to participate in the study, after agreement by parents and the Ethical Committee, but only 36/60 children were recruited (**Figure 3.5**). The most frequent reasons for non-recruitment included parental refusal and unavailability of parents. The recruited children were followed up during two RSV seasons and both NPA and sequential serum samples were collected as often as possible (by asking the parent to bring their children to the hospital whenever they had any flu like symptoms) as summarized in **Appendix 3.** Four sequential sera were available (acute, after 1 month, after 1 year and after 1 year and 1 month) for four children, three sequential sera were available (acute, after 1 month and after 1 year) for fifteen children and the rest of Qatar study children 2 sequential sera only were available (acute and after 1 month). Unfortunately no reinfection episode or isolate was detected among the Qatar study population (**Figure 3.6**).

Figure 3.5: The plan of laboratory work using the clinical samples.
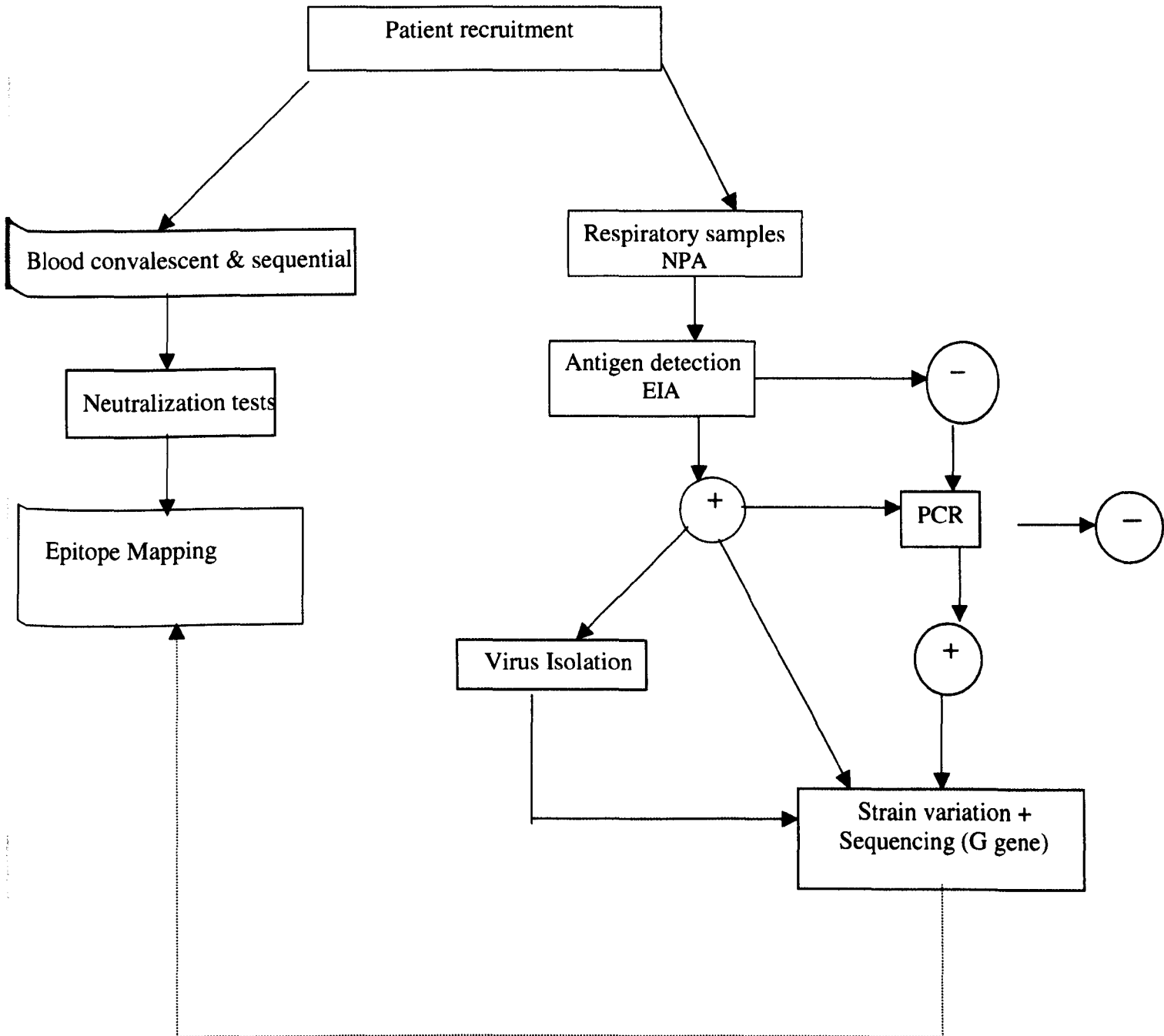
Figure 3.6: Schematic diagram indicating of the start and end of each season during the study period.

| November 1999 | December | / | January | / | February | / | March | / | → | April 2000 |

<div style="color:blue">A/Q/1000</div>
<div style="color:red">B/Q/11/00</div>
<div style="color:blue">A/Q/14/00</div>

A/Q/1000
B/Q/11/00
A/Q/14/00

A/Q/21/00
A/Q/26/00
A/Q/27/00
B/Q/28/00

B/Q/31/00
B/Q/33/00

| December 2000 | / | January | / | February | / | March | / | → | April 2001 |

A/Q/37/01

A/Q/39/01

A/Q/41/01
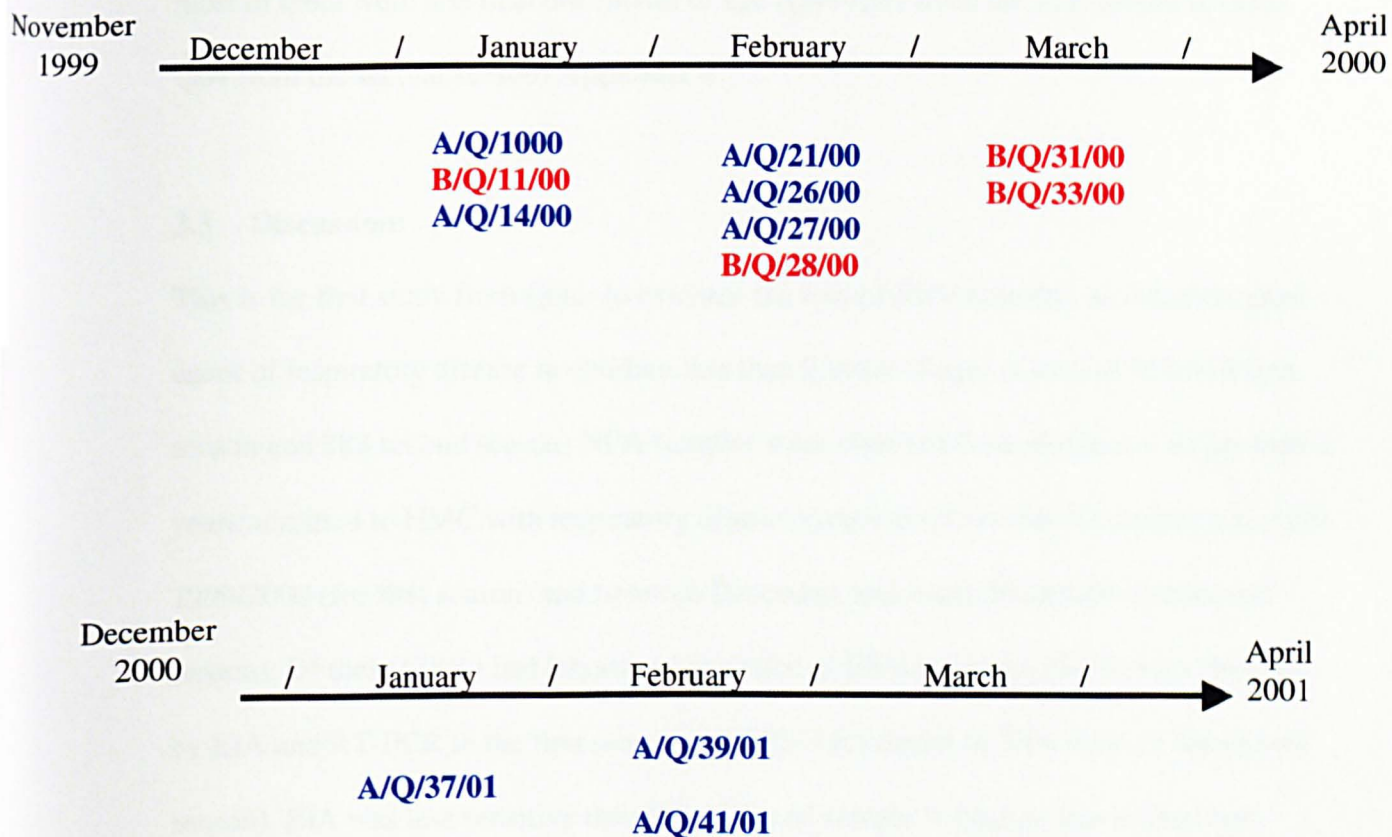
5 RSV A isolates were obtained during the first season (A/Q/10,14,21, 26, 27/00) and 3 from the second season (A/Q/37,39,41/01), while all RSV B isolates were obtained from the first season (B/Q/11,28,31,33/00). Note that there is no reinfection. Blue and red colours indicate the availability of RSV A and B isolates.

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3.2.7   Neonatal samples

In order to validate the Qatar sample sera 20 Qatar sera were collected at random from children admitted to HMC with non-respiratory symptoms during the same study period, most of them were less than one month of age (Q44-Q54 from the first season & Q55-Q64 from the second season) **Appendix 4**

3.3   Discussion:

This is the first study from Qatar to examine the role of RSV infection as the etiological agent of respiratory disease in children less than 2 years of age. A total of 801(418 first season and 383 second season) NPA samples were obtained from children younger than 2 years admitted to HMC with respiratory disease symptoms, between November and April 1999/2000 (for first season) and between December and April 2000/2001 (for second season). Of these (38%) had laboratory evidence of RSV infection, (54 % were detected by EIA and RT-PCR in the first season and 20% were detect by EIA only, in the second season). EIA was less sensitive than RT-PCR and sample collection was started one month later in the second season. This finding indicates that RSV infection was the major infectious cause of hospitalisation of children with respiratory symptoms during the study period in Qatar. This finding is similar to reports from Europe (Whitehead et al., 1999), the United States (Halstead & Jenkins, 1998) and some developing countries (Weber et al., 1998; Maitreyi et al., 2000). The detection rate (per cent) of RSV infection in the Arabian Gulf and other countries (summarized in **Table 3.4**), as assayed by ELISA or culture detection methods, varied from 29% in the United Arab Emirates (Uduman et al., 1996), 40% in Kuwait (Hijazi et al., 1995) and 79% in Saudia Arabia (Bakir et al., 1998). Recently RSV was found to be a major pathogen causing acute lower respiratory tract infections in India, being detected in 24.5% of children by RT-PCR (Rajala et al., 2003),

and a large proportion (45%) of RSV cases associated with pneumonia in Native Alaskan

children (Bulkow et al., 2002). One study from Mozambique indicated that RSV

infection was present in 8.6% of study infants in the outpatient department and 10.6% of

admitted children with LRTI (Loscertales et al., 2002).

It can be seen from Table 3.4 that there is a big variation in the percentage of RSV

detection. This variation may be due to the age of the children who were tested. The

frequency of RSV detection would also depend on the sensitivity of the method used, the

type of sample (NPA or nasal swab) tested, the timing of sample collection after the onset

of infection or whether there is any other infection present in the sample which might

interfere with RSV detection. It is interesting that infectious enteric disease appears to be

a minor cause of hospital admission in this study, despite the fact that Qatar is considered

a developing country where diseases due to enteric infection usually are a major cause of

admission of children to hospital (Wright 1996). A possible explanation is that Qatar is

relatively affluent compared with many other developing countries and has good health

facilities.

Several studies have found a predominance of male patients suffering respiratory illness,

which corresponds generally to the higher incidence of ALRI of any aetiology in boys

(Weber et al., 1998; Hijazi et al., 1995; Dawod & Hussain, 1995).

Table 3.5: The detection rate (%) of RSV infection in the Arabian Gulf and other countries.

Country	Method of detection	% Positive (N)	Type of Specimen	Age group	Reference
UAE	* EIMFA	29% (252)	+ NPW	< 3 years	(Uduman et al., 1996)
Kuwait	** IFA	40 % (523)	++ NPS	< 13 years	(Hijazi et al., 1995)
Saudi a	IFA	79 % (1429)	+++ NPA	<5 years	(Bakir et al., 1998)
India	*** Rt-PCR	24.5 % (200)	NPA	7 – 60 months	(Rajala et al., 2003)
Mozambique	EIA (ABBOT)	10.6% (4589)	NPA	< 5 years	(Roca et al., 2001)
Alaska Native children	Tissue culture or EIA	45 % (431)	NPA	< 3 Years	(Bulkow et al., 2002)
Jordan	IFA	25.46 % (271)	NPW	< 2 years	(Bdour, 2001)
Argentina	**** IIF	36.3% (168)	NPA	< 2 years	(Videla et al., 1998)
Hong Kong	Tissue culture or EIA	13.9 % (1340)	NPA	< 5 years	(Chan et al., 1999)
Iraq	EIA (ABBOT)	37.6 % (57)	NPA	< 5 years	(Albargish & Hasony, 1999)
Qatar	EIA (ABBOT) & RT-PCR	38% (801)	NPA	< 2 Years	1999/2000 & 2000/2001

(N) Total study number
* Enzyme immuno membrane filter assay (EIMFA)
** Immuno fluorescence antibody (IFA)
*** Reverse transcription – PCR
**** Indirect Immuno fluorescence antibody (IIF)
+ Nasopharyngeal washing
++ Nasopharyngeal swap
+ ++Nasopharyngeal aspirate

Comparisons between RSV incidences, detection method used and age in work published from various developing countries. The detection methods have some effect on the percentage of RSV detection.

Although asthma was present in a few cases at HMC (8.9 % as secondary classification), a previously published study (Dawod & Hussain, 1995), which examined childhood asthma in Qatar, found that viral respiratory infections were the most common (95%) precipitating factors for asthma, and 44% of children with RSV bronchitis had further episodes of wheezing over the next two years. In addition, in the current study, RSV detection was highest among the youngest children (0-3 months). Therefore, during the months of the October to March, RSV is an important pathogen in infants and young children in the state of Qatar, highlighting the need for the development of effective vaccines (Wahab et al., 2001).

In most published studies, RSV has been a highly seasonal infection and peaks during winter with an annual peak in November, December and January in temperate climates and during the rainy season in warmer climates (Weber et al., 1998b). This information it important for healthcare provision and implementation of RSV prevention (Chan et al., 2002). In Qatar, a desert country, the pattern of RSV infection showed a clear seasonality during winter months despite the fact that winter is mild with little rain. A similar pattern was observed in other Arabian Gulf countries (Uduman et al., 1996; Hijazi et al., 1995; Bakir et al., 1998; Wahab et al., 2001). Moreover RSV incidence was found to correlate with high relative humidity and low temperature (**Figure 3.2**). A possible explanation is that these conditions allow the optimum survival of RSV in this environment. These results are consistent with those obtained by Chan in Hong Kong (Chan et al., 1999). However similar studies in Singapore (Chew et al., 1998) do not show a relationship with humidity. Despite the fact that humidity in the summer in Qatar is usually at approximately the same high level or higher than in winter, the temperature is also very high (~ 45°C). It is interesting that a phenomenon of increasing virus yield in vitro with lower temperatures has been recorded using different cell lines and detection methods

e.g. virus growth on Hep2 detected by immunolocalization of infected cells (Razinkov et al., 2002) and the virus growth on human airway epithelial cells detected by an immunofluorescence assay (Zhang et al., 2002). Ambient temperature in Qatar may be sufficiently high to inactivate virus. Parental smoking and the use of wood burning stoves have been shown to increase the risk and severity of RSV infections. It is important to define the role of air humidity or temperature, either indoors or outdoors, or of industrial pollution, which could also affect the capacity of young infants to cope with respiratory infections (Brandenburg et al., 1997).

The success of detection of respiratory viruses depends on the type of specimen collected, transport of specimen and method of virus isolation. In this study NPA specimens were chosen for virus isolation. NPA yields a greater number of cells for virus isolation (Maitreyi et al., 2000) and collection is also a less traumatic procedure for young children. A study in Glasgow tested the sensitivity of perinasal swabs in comparison with NPAs and reported 93% and 99% sensitivity, respectively (Mackie et al., 1991). By comparing nasal swabs (NS) with NPA or nasal wash, previous studies from developing countries (Stensballe et al., 2002), found between 9 and 65% reduction in sensitivity. Also, in anothr developing country, using a nasal swab was associated with a 27 – 31 % reduction in sensitivity compared with a NPA (Stensballe et al., 2002). Therefore, NPA appears to be the ideal specimen for RSV detection. However, collecting NPA specimens is costly and requires a vacuum system, a suction catheter, a mucus trap and transport medium. NS collection only needs a swab and a transport medium and it might be preferable in epidemiological studies, where a large number of samples are reqiuered.

In the work described here, multiplex PCR has been shown to be a more reliable tool for the diagnosis of RSV infection than antigen detection methods, especially in young children. The 9 samples positive by EIA test, which were negative by multiplex PCR,

could be explained by either a false positive result in the EIA or viral genome degradation before PCR, as sample analysis was performed several months after collection. In addition, antigen detection cannot be used for RSV sub typing analysis. A further advantage of using the PCR method is for the detection of dual infections, of which there were minorities in each season (16 in the first season and 3 in the second season and one RSV A and Flu B in the first season). It is not known to what extent the two viral genomes (mixed infection) detected contributed to the disease symptoms of the patients (Lina et al., 1996; Drews et al., 1997). The RT-PCR technique is a very sensitive method, which may be capable of detecting viral genomes for a period of time after symptoms have abated. This finding draws attention to the primary cause of illness and to the potential of one respiratory illness to predispose to another. There is very little information about RSV A and B dual infections because most studies do not subtype RSV strains. However, Drew et al (Drews et al., 1997) noticed that RSV was associated with most dual infections with other viruses, a finding confirmed by this present work where the majority of dual infections were associated with RSV A and B.

Another important seasonal viral infection, which was detected in the study population during first season, was influenza virus that could be detected and sub typed in the multiplex PCR that was used for RSV detection. The finding of low influenza infection among children under two years of age during the study period agreed with other studies (Sugaya et al., 2000; Maitreyi et al., 2000). This could be explained because infants may be protected from influenza virus infection by passive maternal antibodies, whereas high RSV incidence in infants indicates that RSV infection can occur despite the presence of maternal antibodies (Glezen et al., 1981).

Rapid diagnosis of respiratory viruses is essential for initiating immediate appropriate therapy (e.g. antiviral drug), reduces the use of unnecessary antibiotics and is important

in the hospital management of the patient (Maitreyi et al., 2000; Savon et al., 2000). The EIA used here showed a reasonable sensitivity (74%) and specificity (95.5%) as has been found by others (Dayan et al., 2002). It appears that the Abbott Testpack® is a useful tool in the detection of RSV in febrile infants but it has limitations. During months typically associated with RSV disease, a positive RSV Testpack® indicates a high likelihood of illness, but clinicians should be wary of false negatives.

The current work defined the incidence of RSV infection (38%) among 801 children under 2 years of age admitted to HMC with respiratory disease symptoms. Systemic analysis of specimens for pathogens other than this virus was not undertaken and therefore the possibility of an important contribution of other respiratory viruses in the remaining negative samples (62%) cannot be ruled out nor can their possible contribution to dual infections among RSV positive samples (38%). The significance of RSV infection in other age groups or in other infections with other respiratory viruses, which are common cause of morbidity, mortality, and loss of productivity, also needs to be determined.

4 Chapter4: Molecular analysis of the diversity of the G protein gene from Qatar RSV isolates

4.1 Introduction:

All Pneumoviruses encode two major surface glycoproteins (G and F), which are incorporated into the virus particle. The G protein has been shown to be the most divergent gene, which distinguishes between subtypes A and B and also between genotypes within these subtypes. There is 67% similarity at the nucleotide level and 53% similarity at the deduced amino acid level between group A and B strains (Peret et al., 1998). This protein is responsible for the attachment of virus particles to the cell receptor. It is a type II glycoprotein with a single hydrophobic domain between residues 38 and 66 that serves as a transmembrane anchor. The G protein precursor is synthesized as a 32-Kda single peptide, which is modified by the addition of O-linked and N-linked sugar moieties to give the mature form with a molecular mass of 80 to 90 Kda. The protein ectodomain contains four cysteine residues (codons173, 176, 182, and 186), which are conserved in all RSV isolates and a short segment (residues 164 to 176) of exact sequence identity between the two antigenic subgroups (Garcia et al., 1994) or in all group A isolates (amino acids 163- 189) (Martinez et al., 1997). In addition, the G protein is produced in two forms, anchored G protein and soluble G protein, which are produced by translation and processing from different start codon (AUG) (Anderson et al., 1995). Three types of epitope have been identified in the G protein by testing the reactivity of monoclonal antibodies with a large panel of viral strains: (1) conserved epitopes that are present in all RSV isolates, (2) group specific epitopes shared by all RSV strains of the same antigenic group, and (3) strain-specific or variable epitopes that are present in certain isolates of the same antigenic group (Melero et al., 1997). The strain-specific epitopes have been mapped within the hypervariable C-terminal third of the G protein

ectodomain. It has been shown that the C-terminal region of the RSV G protein includes epitopes important for the neutralization of the virus and that loss of the terminal amino acid can allow escape from antibody neutralization(Cane et al., 1992). Epitopes of the other two types have been tentatively located near the four-cysteine cluster of the protein ectodomain (Garcia et al., 1994; Cane & Pringle, 1991) have shown that the amino acid variability is not evenly distributed over the G protein; in the extracellular part of the protein there is a sharply defined hypervariable domain separated from a more extended variable domain by a highly conserved region.

The genetic variability of RSV has been best characterized for group A strains. Up to 20% diversity have been shown at the deduced amino acid level among group A strains and 9% within group B (Cane et al., 1996). There is an additional G protein variability within antigenic groups with amino acid differences of as great as 20% in a single epidemic period. This exceeds the 10% differences among the heamagglutanin (HA) protein of drift strains of influenza A virus. G-protein mutations are due to frame shifts, premature stop codons, substitutions, and hypermutation (Sullender & Edwards, 1999). The reason for this diversity is not completely clear.

It has been demonstrated that genetically similar strains seem to cluster temporally rather than by geographical location (Cane & Pringle, 1991). These studies suggest that immunological pressure may direct RSV evolution. Although such studies have provided important insights into the epidemiology of RSV strains, the circulation pattern of distinct strains in a single community and simultaneously in different communities have still not been fully characterized (Peret et al., 1998). Limited strain diversity studies are available from the developing world.

RNA viruses (for example RSV or Influenza A and B) show a high level of genetic divergence. This can be observed by the emergence of variant strains in the population.

The level of genetic divergence among the G glycoproteins of RSV virus is the highest reported for an RNA virus gene product (Garcia et al., 1994). It is possible that RSV follows the evolutionary patterns of influenza A & B viruses. It remains to be determined whether or not immune selection is a factor determining genetic variation and evolution of RSV from the same antigenic subgroup. To clarify this point both the antigenic characterization of clinical isolates and sequence analysis of the relevant genome segments should be carried out (Cristina et al., 1991; Akerlind et al., 1988). If this is coupled to evaluation of antibody responses to known genotypes, particularly in primary infection, it is a powerful tool for dissection of RSV strain variation and the forces driving it.

The aim of the work described in this chapter was to evaluate the genetic diversity of both group A and B RSV strains by sequencing the G protein gene of isolates from young children collected during two seasons in a single community in Qatar. In this way it was hoped to obtain a series of RNA sequences of Qatari strains to compare with available RNA sequences of RSV in GenBank. The aim was also to provide information about the diversity of the RSV B strain and the selection of the RSV B strain for use in epitope mapping.

4.2 Results:

4.2.1 Strain variation:

Multiplex PCR detection allowed differentiation of RSV into RSV A and RSV B subtypes as shown in **Figure 3.4.** RSV A strain was detected in more than 60% of all RSV infections in all age groups sampled for 2 seasons, a small proportion of dual infections with RSV A and B were also detected in all age groups. The PCR detection strategy for RSV is based on amplification of the N and P region of the genome; this was selected because it is highly conserved between strains and also allows subtyping of RSV

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into A and B subtypes (Stockton et al., 1998). However, to derive sequence analysis of G gene, which is highly variable, required a different PCR detection strategy. Accordingly, clinical material that was found to be positive for RSV by PCR and EIA, was used as source material to attempt to isolate RSV and derive sequences, in accordance with the flow chart shown in **Figure 3.5.** Cases for whom paired sera, as well as an NPA sample were available, were used to isolate RSV. Using this approach, a total of 36 NPAs, known to contain RSV by PCR and EIA, were inoculated into Hep 2 cells. A total of 12 RSV isolates (8 of strain A and 4 of strain B) were prepared and their subtype properties were defined (**Appendix 3 and Figure 4.1**).

4.2.2   Amplification of the G gene:

4.2.2.1   G gene PCR optimization:

There were many problems with amplification of the G gene directly from clinical material, including the high degree of variability of the gene making it difficult to design primers. There are also a limited number of published sequences especially for RSV B therefore enhancing problems for primer design.

The overall strategy involved designing primers which would amplify the whole G gene (923 nucleotides) in a single reaction, including a small amount of additional upstream sequence from the SH gene (SH 136 Forward) and downstream sequence from the F (F1Reverse) gene. This primary product could be used to generate secondary overlapping fragments, which could be used in sequencing reactions.

Figure 4.1: Flow chart of sequencing materials, both NPA and cell culture grown virus from Qatar children group were used to generate RNA sequences of Qatar strains.

5 complete sequences of RSV A were obtained from viral isolates (A/Q/ 10,14,27/00 and A/Q/ 39, 41/01) and 5 were obtained from NPA samples (A/Q/ 13,16,17,19,29/00). 3 complete sequences of RSV B were obtained from viral isolates (B/Q/11, 28, 33/00) and 2 were obtained from NPA samples (B/Q/5, 18/00).

Sources of sequence which were used to design primers which could amplify the whole of the G gene were those described in the following publications: (Wertz et al., 1985; Cane & Pringle, 1991) for RSV A and (Roca et al., 2001; Sullender & Edwards, 1999; Taylor et al., 2001) for RSV B. There were limited numbers of sequences available to choose conserved regions.

Primers designed to amplify the smaller internal overlapping fragments were chosen in a similar way. The primer sets were designed to be as similar as possible in their physical and chemical characteristics, around 20 bases in length, with a 50% G+C content (**Table 4.1.**).

Primers were designed to sequence the whole Human RSV G gene (923 nucleotides), in overlapping segments each between 300 -500 base pairs long as described in the materials and methods (section 2.7 & 2.8). The primer mapping positions are demonstrated in **Figure 4.2** and which primer sites yielded product of the expected size is detailed in **Table 4.2.**

Systematic testing of primers was performed because it was not possible to amplify the complete regions of RSV G gene and to give the expected product size was performed using A/Qatar/RSVA/2000/10 for RSV A & RSV B control (N2) for RSV B strains.

Table 4.1: List of the designed primers used in the amplification of the RSV G gene.

Primer name	Sequence 5`---3`	Annealing Temp C°.	GC content %
F1 R	CAA CTC CAT TGT TAT TTG CC	40	40
SH 136F	CAT CCA TAA CAA TAG AAT TC	30	30
G2 F	GGG CAA ATG CAA ACA TGT CCA AAA	41.7	43.5
G152 F	TTG CAA TGA TAA TCT CAAC	31.6	31.5
G178 F	ATA ATT GCA GCC ATC ATA TTC	33.3	33.3
G508 R	AAG TTG AAC ACT TCA AA	29.4	30
G618 R	TTT GTG GGC TTG GTG GTG	55.6	55.5
G645 R	TTT TTT GGT TGT CTT G	31.2	31.2
F95 R	TAA AAT TCT TCA GTG AT	23.5	23.5
G618 F	CAC CAC CAA GCC CAC AAA	55.6	47.6
G645 F	CAA GAC AAC CAA AAA A	31.2	31.2
G508 F	TTT GAA GTG TTC AAC TT	29.4	29.4
SH237 F	ATT CCA TAA CAA AAC CTT	27.8	27.8
G178 R	GAA TAT GAT GGC TGC AAT TAT	33.3	33.3
G248 F	CTT GAT CTG GCT TGT TGC ATC	47.6	47.6
G152 F	ATA ATT GCA GCC ATC ATA TTC	41.7	33.3
G178 F	AAG TTG AAC ACT TCA AA	31.6	33
G618 R	TTT TTT GGT TGT CTT G	29.4	31.2
G645 R	TAA AAT TCT TCA GTG AT	55.6	23.5
* G1F	GGG GCA AAT GCA ACC ATG TCC AAA	72	50
* G390 R	GTT TGT GCT GTT GTA TGG TGT GTT	68	41.6
* G390 F	AAC ACA CCA TAC AAC AGC ACA AAC	68	41.6
G510 F	ACT TTG AAG TGT TCA AC	45	35.2
* G600 R	TGT GGG TTT GAT GGT TGG TTT TTT	66	37.5
* G600 F	AAA AAA CCA ACC ATC AAA CCC ACA	66	37.5
* BG801 R	AAG TTC AAC ACT TCA AA	44	29.5

*: These primers were designed for the current study.

The red primers were used in the generation of the primary product, while blue primers, were used in the secondary reactions.

Figure 4.2 &Table 4.2: Schematic diagram and tables of primer sets used to amplify and sequence the G gene.
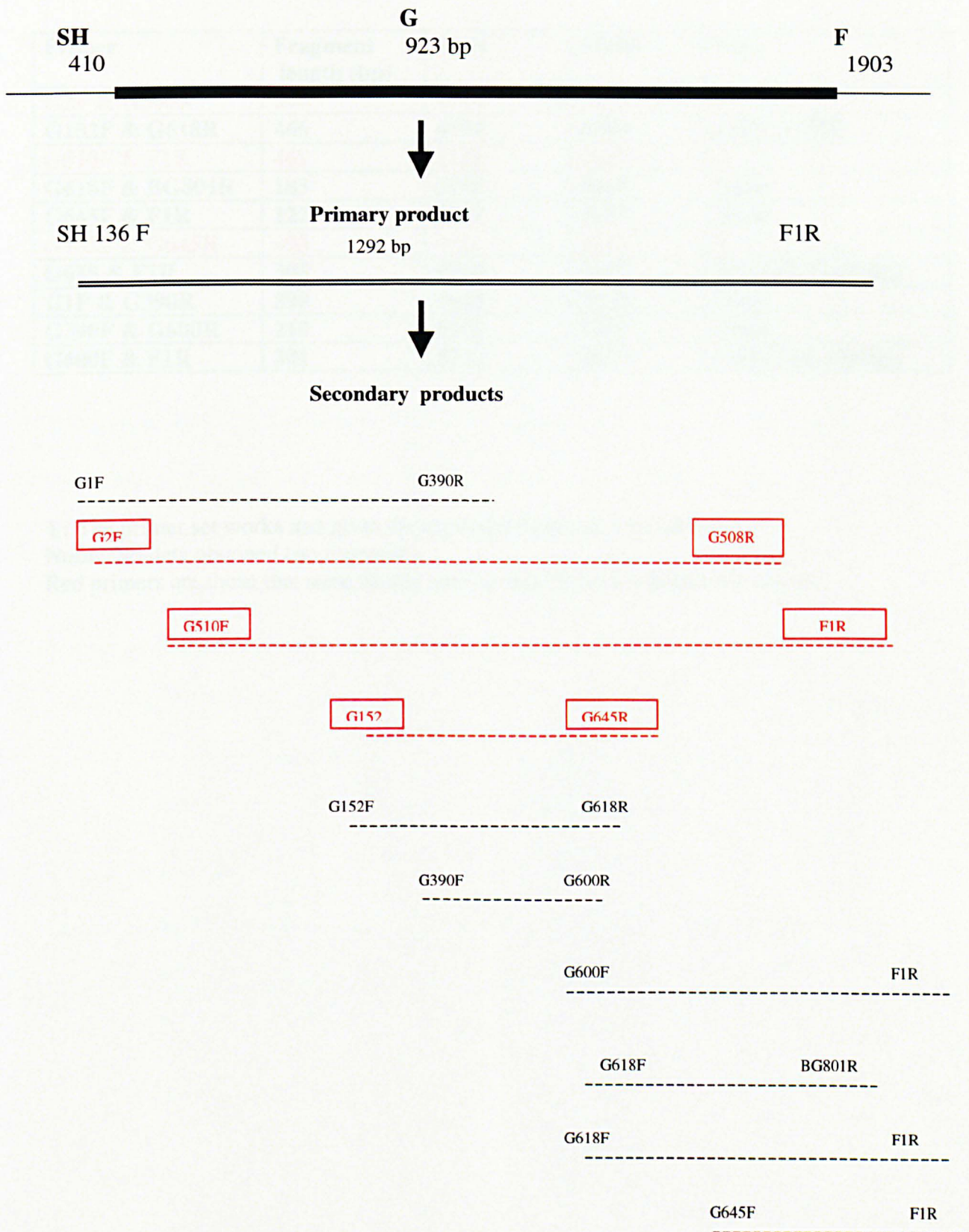
Table 4.2: primer sets used to amplify and sequence the G gene.

Primer	Fragment length (bp)	Start	Finish	Seq.
G2F & G508R	506	4644	5150	Y
G152F & G618R	466	4794	5260	Only G152F
G510F & F1R	465	5152	5617	Y
G618F & BG801R	183	5160	5443	None
G645F & F1R	122	5287	5617	None
G152F & G645R	493	4794	5287	Y
G618 & F1R	305	5260	5617	Only F1R (400bp)
G1F & G390R	390	4643	5032	None
G390F & G600R	210	5032	5242	None
G600F & F1R	301	5242	5617	Only F1R (400bp)

Y: The primer set works and gives the expected fragment with sequence.
None: No data obtained (no fragment).
Red primers are those that were finally used to amplify and sequence the G gene

Some primers sets were excluded for inability to amplify a fragment of the expected size. The reasons for this failure were not fully investigated. Once it had been determined that each primer set yielded a fragment of the expected size, optimization of the reaction conditions was performed, to improve the sensitivity of detection. Biochemical optimization using a range of buffers and pH conditions and annealing temperatures was undertaken. This was done to ensure that the primer sets chosen eventually for sequence analysis would amplify directly from clinical material, which might contain a low copy number of template. Initial approaches to this included comparing the sensitivity of detection of RSV with the multiplex PCR primers with the newly selected G gene primer sets. This optimization was performed using A/Qatar/RSVA/2000/10 virus.

It appeared that the RSV G gene based primers were less sensitive than the NP primers by 10 to 100 fold in their detection of RSV (**Figure 4. 3**). However, biochemical optimisation using different buffer compositions indicated that the sensitivity for detection of RSV could be improved for some of the primer sets (**Figure 4.3**). This figure shows those multiplex PCR primers more sensitive than G gene primers.

4.2.2.2 G gene PCR:

G gene nested PCR conditions were modified from the RT-PCR conditions and the final conditions for G gene amplification are detailed in **Figure 4.3.**

Figure 4.3: Comparison of (a) multiplex PCR (344 bp) and (b) RSV G primers PCR (fragment size 356 bp) amplification.
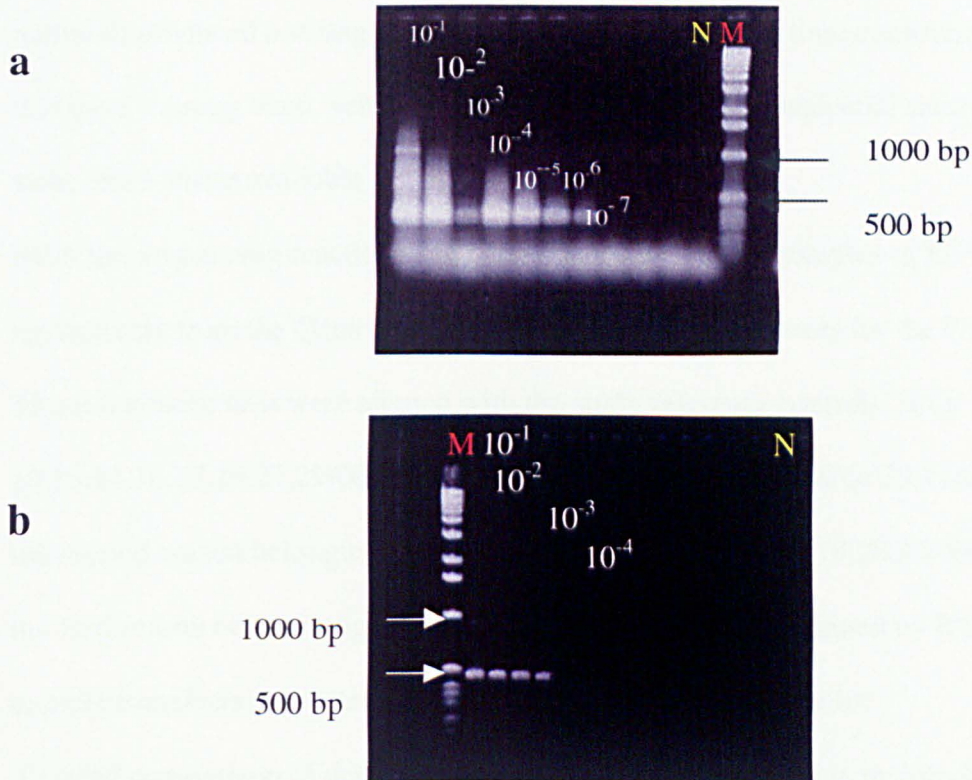
10x dilution series of Qatar /A/2000/10 samples were used. N is a negative control. M is a marker with base pair sizes indicated with arrows.

GPCR conditions were modified from multiplex PCR condition. The primary cycle became as following: 94°C for 2 min x 1, followed by 40 cycles of: 94°C for 1 min, 52°C for 2 min and 72°C for 3 min. While the secondary PCR Cycle became as following: 94°C for 2 min also for 1 minute, but followed by 40 cycles of: 94°C for 1 min, 60°C for 1 min and 72°C for 1 min. The amount of 1°product was also increased from 2µl to 4µl.

4.2.3 Sequence Analysis:

Attempts were made to amplify all of RSV A&B positive materials, either NPA or tissue

culture (if available) from the Qatar study children group, but after purification

procedures only 17 RSV A & 9 RSV B samples (18 from NPA and 8 from tissue culture

material) produced a strong signal (**Figure 4.1**). Because of time constraints, only isolates

that gave a strong band were sequenced with emphasis on sequential samples from the

same child where available.

After the sequencing reactions had been done, only 10 & 5 samples of RSV A &B,

respectively from the Qatar group produced complete sequences for the G gene.

These sequence data were aligned with the study reference controls. A/Q/

10,13,14,16,17,19,27,29/00 isolates from the first season and A/Q/ 39,41/01 isolates from

the second season belonging to the RSV A subtype. B/Q/5,11,18,28,33/00 isolates from

the first season only belonged to the RSV B subtype (as determined by RT-PCR). So,

sequence analysis supported the initial RT-PCR sub typing results.

Limited comparison of derived sequences of the second variable region of the G gene for

A/Q/ 10/00 obtained either directly from clinical material (NPA) or from tissue culture,

indicated that there were no changes at the nucleotide or amino acid level following a

single passage in Hep- 2 cells.

To accurately define the extent of genetic variability within and between groups, the

nucleotide sequences of the complete, partial and C-terminal end of the G protein gene

were determined (10 of group A and 5 of group B) collected throughout the study period.

The sequences determined correspond to nucleotide 1 to 900 of the A2 prototype strain

and to nucleotide 1 to 915 of the N2 strain (this include some areas at beginning and end of the gene that are covered by primers).

There were some coding and non-coding nucleotide substitutions especially for the first and third nucleotide sequence (1-450 and 650 to the end) for both strains (**Figure 4.4** and **Appendix 5**). No insertion, frame shift mutations or up and down stream sequence changes were identified in these Qatar RSV A and B strains. The middle conserved region (450 – 650) was identical among Qatar's RSV A strains with 8 nucleotide substitutions between A2 strain and Qatar strains at positions 468, 506, 515, 552, 558, 611, 639 and 644 (noncoding), whereas there were 12 nucleotide substitutions in the middle region of RSV B strains at positions 470, 476, 508, 559, 573, 591, 603, 627, 634, 638, 643, 646 and 650 which were non synonymous.

The nucleotide variation detected was 5% within Qatar group A isolates (complete sequences) and 15% between Qatar and non Qatar isolates for the partial sequences and 4.6 & 13%, respectively for the nucleotide sequences of the C-terminal end of the G protein gene at the nucleotide level.

The nucleotide variation observed within the group B isolates from Qatar was 0.8% (complete sequences). The mean nucleotide variation between Qatar and non-Qatar isolates was 10% for the partial sequences and 2.3% & 12.5%, respectively for the nucleotide sequences of the C-terminal end of the G protein gene at the nucleotide level.

Figure 4.4 (a&b): The deduced amino acid alignment by Clustal V method of Qatar RSV strains

```
                 10        20        30        40        50        60        70        80        90       100       110
A2        : MSKNKDQRTTKTLEKTWDTLNHLLFISSCLYKLNLKSIAQITLSILAMIISTSLIIAAIIFIASANHKVTLTTAIIQDATSQIKNTTPTYLTQNPQLGISFSNLSETTSQ
A/Q/10/00 : ...T.....A...R.................................................................N................D...............
A/Q/13/00 : ...T.....A...R................FF.............................................N..............................
A/Q/14/00 : ...T.....A...R................FF.............................................N..............................
A/Q/16/00 : ...T.....A...R................F..........................M...................N..............................
A/Q/17/00 : ...T.....A...R................FF.........................................N..................................
A/Q/19/00 : ...T.....A...R..........................................M...................N..............................
A/Q/27/00 : ...T.....A...R..............................................................N..............D...............
A/Q/29/00 : ...T.....A...R..............................................................N..............D...............
Q/A/39/01 : ...T.....A...R..............................................................N.......................I...
A/Q/41/00 : ...T.....A...R..............................................................N.......................I...
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                120       130       140       150       160       170       180       190       200       210       220
A2        : TTTILASTTPSVKSTLQSTTVKTKNTTTTKIQPSKPTTKQRQNKPPNKPNNDFHFEVFNFVPCSICSNNPTCWAICKRIPNKKPGKKTTTKPTKKPTIKTTKKDLKPQTT
A/Q/10/00 : PI.........AEL.P.....I......QM.................Q...............................................P.....
A/Q/13/00 : P..........AE..P.....I......Q..................Q..............................................P.....
A/Q/14/00 : P..........AE..P.....I......Q..................Q..............................................P.....
A/Q/16/00 : PI.........A...P.....I......QM.................Q.........................................R.........P.....
A/Q/17/00 : P..........AE..P.....I......Q..................Q..............................................P.....
A/Q/19/00 : PI.........A...P.....I......QM.................Q.........................................R.........P.....
A/Q/27/00 : PI.........AEL.P.....I......QM.................Q..............................................P.....
A/Q/29/00 : PI.........AEL.P.....I......QM.................Q..............................................P.....
Q/A/39/01 : P..........AE..P.....I......Q..................Q....................................................
A/Q/41/00 : P..........AE..P.....I......Q..................Q....................................................
```

```
                230       240       250       260       270       280       290       298
A2        : KPKEVPTTKPTEKPTINTTKTNIRTTLLTNNTTGNPEHTSQKGTLHSTSSDGNPSPSQVYTTSEYLSQPPSPSNTTNQ
A/Q/10/00 : .....L....................K.....S...E........E.....T.E................SL......KL
A/Q/13/00 : .....L....................P.............E.....T.E................S......K-
A/Q/14/00 : .....L....................P.............E.....T.E................SL.....KL
A/Q/16/00 : .....L.......R............K.....P.......E.....T.E................SL......K-
A/Q/17/00 : .....AL..................P..............E.....T.E................S......E-
A/Q/19/00 : .....L.......R............K.....S.......E.....T.E................SL......K-
A/Q/27/00 : .....L....................K.....S...E...E.....T.E................SL......KL
A/Q/29/00 : .....L....................K.....S...E...E.....T.E................SL......K-
Q/A/39/01 : ...VALS.......S....R.S....M..S..........E.....T.E................S........W
A/Q/41/00 : .....LS.......S....R.S....M..S..........E.....T.E................S......K-
```

(a) The deduced amino acid alignment by Clustal V method of Qatar RSV A strains, with total length of 297 and 298 amino acid for all viruses.Red boxes indicate the strictly conserved region in both RSV strains (A & B).

```
                 10        20        30        40        50        60
BN2      : MSKHKNQRTARTLEKTWDTLNHLIVISSCLYRLNLKSIAQIALSVLAMIISTSLIIAAII
B/Q/5/00 : ..................................K.........................
B/Q/11/00: ............................................................
B/Q/18/00: ............................................................
B/Q/28/00: ..................................K.........................
B/Q/33/00: ............................................................


                 70        80        90       100       110       120
BN2      : FIISANHKVTLTTVTVQTIKNHTEKNITTYLTQVPPERVSSSKQPTTTSPIHTNSATTSP
B/Q/5/00 : .......................................S.....P......P........I..
B/Q/11/00: .......................................S.....P......P........I..
B/Q/18/00: .......................................S.....P......P........I..
B/Q/28/00: .......................................S.....P......P........I..
B/Q/33/00: .......................................S.....P......P........I..


                130       140       150       160       170       180
BN2      : NTKSETHHTTAQTKGRTTTSTQTNKPSTKPRLKNPPKKPKDDYHFEVFNFVPCSICGNNQ
B/Q/5/00 : ....................P..N........P.S..........................
B/Q/11/00: ....................P..........P.S..........................
B/Q/18/00: ....................P...K........P.S..........................
B/Q/28/00: ....................P..N........P.S..........................
B/Q/33/00: ....................P...K.......P.S..........................


                190       200       210       220       230       240
BN2      : LCKSICKTIPSNKPKKKPTIKPTNKPTTKTTNKRDPKTPAKTTKKETTTNPTKKPTLTTT
B/Q/5/00 : ............................P...........L...L.........E...PK..
B/Q/11/00: ............................P...........L...L.........E...PK..
B/Q/18/00: .......................K...I...K.........L.........EE..PK.K
B/Q/28/00: .......................K...I...K.........L.........EE..PK.K
B/Q/33/00: .......................K...I...K.........L.........EE..PK.K


                250       260       270       280       290      299
BN2      : ERDTSTSQSTVLDTTTLEHTIQQQSLHSTTPENTPNSTQTPTASEPSTSNSTQNTQSHA
B/Q/5/00 : .G............AS...V.................................KP----
B/Q/11/00: .G............AS...V.................................KP----
B/Q/18/00: .............AS...V..................................P----
B/Q/28/00: .............AS...V.............................L........RD
B/Q/33/00: .............AS...V.............................L........RD
```

(b) The deduced amino acid alignement by Clustal V method of Qatar RSV B strains, with total length of 295 amino acid for 3 viruses and 299 for the rest. Red box indicate to the strictly conserved region in both RSV strain (A & B) viruses.

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The deduced amino acid sequences of the Qatar group A isolates indicated that all of them had either a deduced G protein length of 297 or 298 amino acid due to presence of a ATG start codon and TAG stop codon.

Two clusters of RSV A 1999/2000 & 2000/2001 (with 8 for first season and 2 for the second season) with similar amino acid sequences within each of them were detected among Qatar group A strains (**Figure 4.4a & 4.5.a**). Sequences from these clusters differed by 35 to 40 amino acid over the sequenced length.

Again two clusters of viruses with similar amino acid sequences were detected among group B Qatar strains (**Figure 4.4.b**). Sequences from these two clusters differed by 20 to 25 amino acids over the sequenced length.

There were some O-glycosylation sites (monosacharide-S/T) especially on the first part (1-160) and the third part for both RSV strain viruses. N-glycosylation sites (carbohydrate-N) were more localised at the C-terminal end than the first part of the G protein of these viruses at the following positions 85, 104, 134, 237, 251, 272 and 293 (7 sites) for RSV A viruses and at 86, 230, 274 and 290 (4 sites) positions for RSV B viruses, these sites were varied between strains. In general, there were fewer non-synymous changes in the first region than in the third part. The almost conserved middle nucleotide region (450-650) in both strains had a few nucleotide substitutions leading mainly to no-change (synonymous change) at the strictly conserved region (160-200) and no change in amino acid sequences of this part of the protein.

Several phylogenetic trees were constructed using windows of different lengths and the longest and recent nucleotide sequences of the G protein gene already available for RSV isolates especially group B with particular emphasis being placed on the C-terminal end of this gene.

The phylogenetic analysis derived by the neighbour-joining method of the complete

nucleotide sequence of Qatar isolates showed that most strains have similar amino acids

(1-298 amino acids) **Figure 4. 5 a & b**. Only bootstrap values (distance/parsimony)

greater than 60% are shown. For the full length of the RSV A strains, the most significant

bootstrapping value is that 100/99 time A/Q/39/01 & A/Q/41/01 are clustered together

from the rest of the isolates used in the analysis, A/Q/10,27,29/00 are clustered 99/95

times, while strains A/Q/16/19/00 are grouped together 93/90 times, strains A/Q/13,14,17

clustered together 84/73 times. All Qatar isolate group together 80% of the time and are

related to A/MAD/92.

For RSV B strains full length the B/QAT/18/00, 33, 28 are grouped together 93/94 times

more than other. B/QAT/18/00 clusters together 93 times with B/QAT/33/00 by using

Distance method although it clusters 62 times with B/QAT/33/00 by using Parsimony

method. B/QAT/5/00 & B/QAT/11/00 strains grouped together 86/88 times. In general

all Qatar B strains are close together.

Figure 4.5 (a & b): Phylogenetic trees of the complete sequence of the RSV G protein gene of Qatar strains
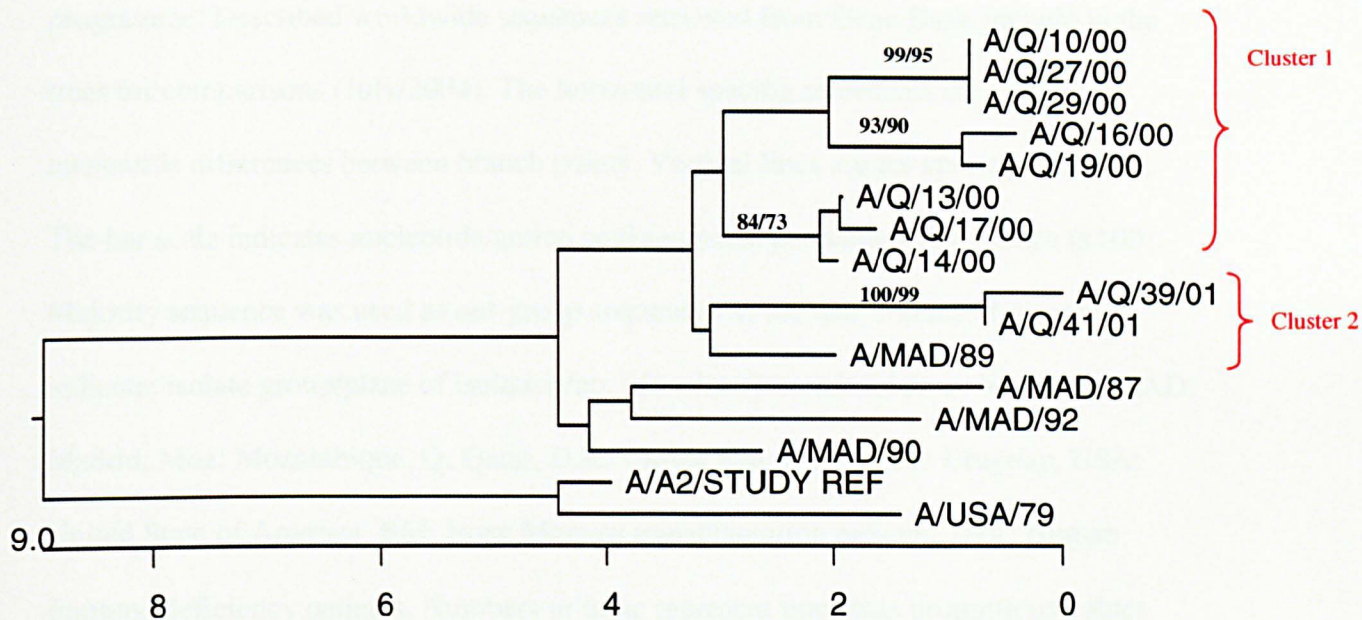
(a) Phylogenetic tree of the complete sequence of the RSVA G protein gene of Qatar other worldwide isolates encompassing amino acid 1 to 298 were included in the analysis.
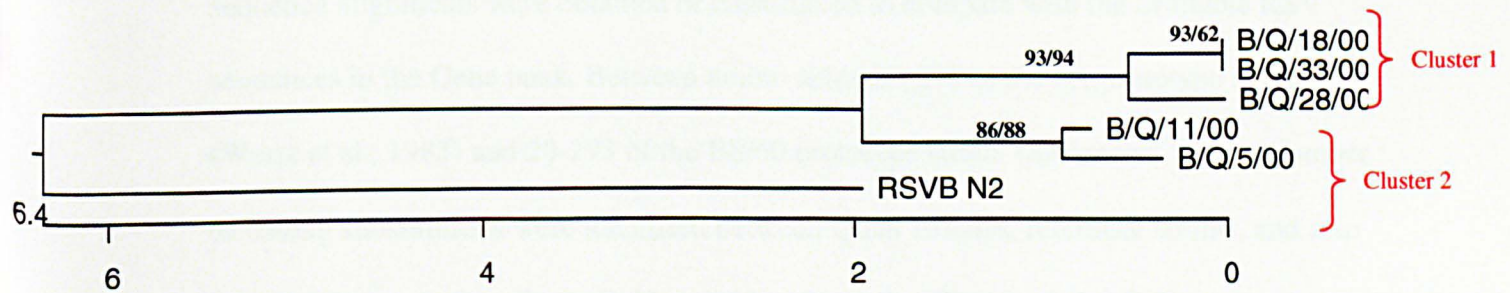
(b) Phylogenetic tree of the complete sequence of the RSVB G protein gene of Qatar & one USA isolate encompassing amino acid 1 to 295 were include in analysis.

Comments on figure 4. 5: The phylogenetic trees of group A & group B RSV isolates were derived by the neighbour-joining method as determined by the PHYLIP computer programme. Described worldwide sequences retrieved from Gene Bank include in the trees for comparisons (July/2004). The horizontal spacing represents the number of nucleotide differences between branch points. Vertical lines are for spacing and label. The bar scale indicates nucleotide/amino acid sequence percentage divergence (x100). Majority sequence was used as out-group sequences in the tree. Names of viruses indicate: isolate group/place of isolation/no. of isolate/year of isolation. K: Korea, MAD: Madrid, Moz: Mozambique, Q: Qatar, U.K: United Kingdom, URG: Uruguay, USA: United State of America, BM: Bone Marrow transplantation patients, HIV: Human Immune deficiency patients. Numbers in italic represent bootstrap proportions values (Distance/Parsimony method). Only bootstrapping values greater than 60% are shown.

In an attempt to compare Qatar isolates with more worldwide isolates, shorter amino acid sequence alignments were obtained or constructed to compare with the available RSV sequences in the Gene bank. Between amino acids 20-296 of the A2 prototype strain (Wertz et al., 1985) and 20-293 of the B8/60 prototype strain (Sullender, 1995) a number of coding substitutions were identified between Qatar isolates, reference strains, and also between the most recently available world wide strains (**Figure 4. 6 a & b**).

Figure 4.6 (a & b): The partial phylogenetic tree, Comparison between Qatar and worldwide isolates (July/2004)
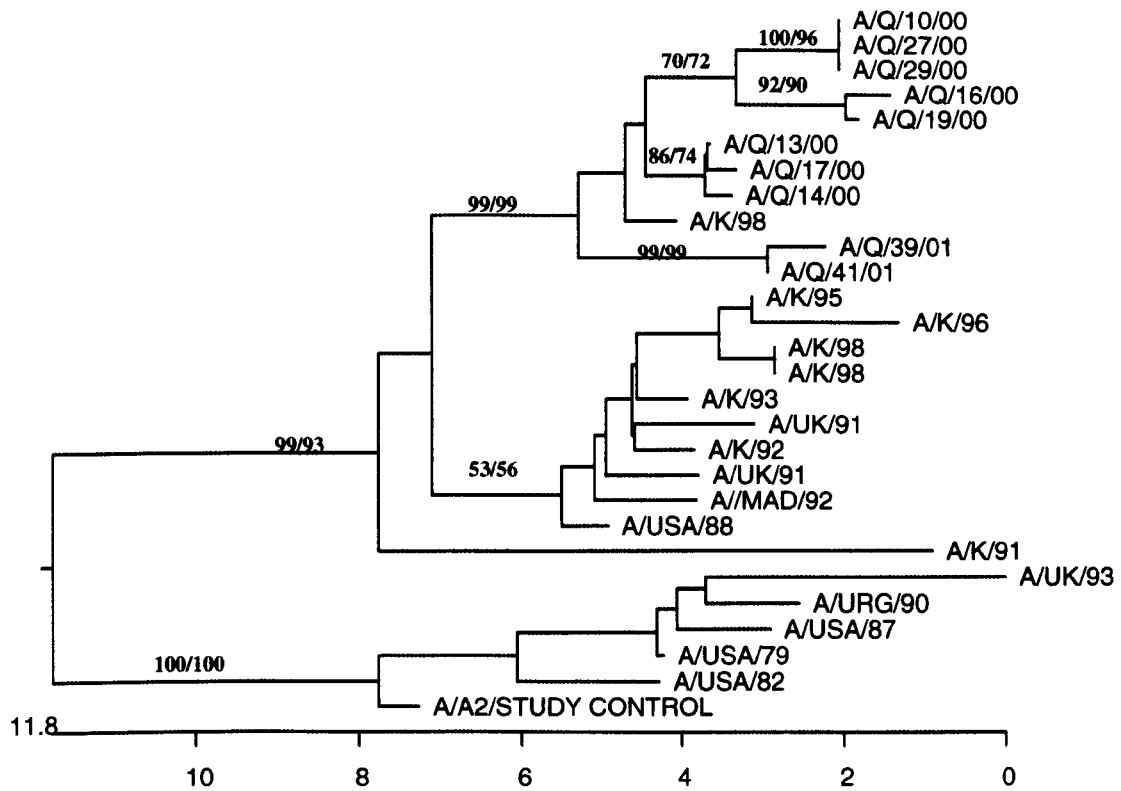
(a) Phylogenetic tree of the partial sequence of the RSVA G protein gene of Qatar & other world wide isolates encompassing amino acids 20 to 296 were included in the analysis.

Figure 4.6:(a & b): The partial phylogenetic tree, Comparison between Qatar and worldwide isolates (July/2004)
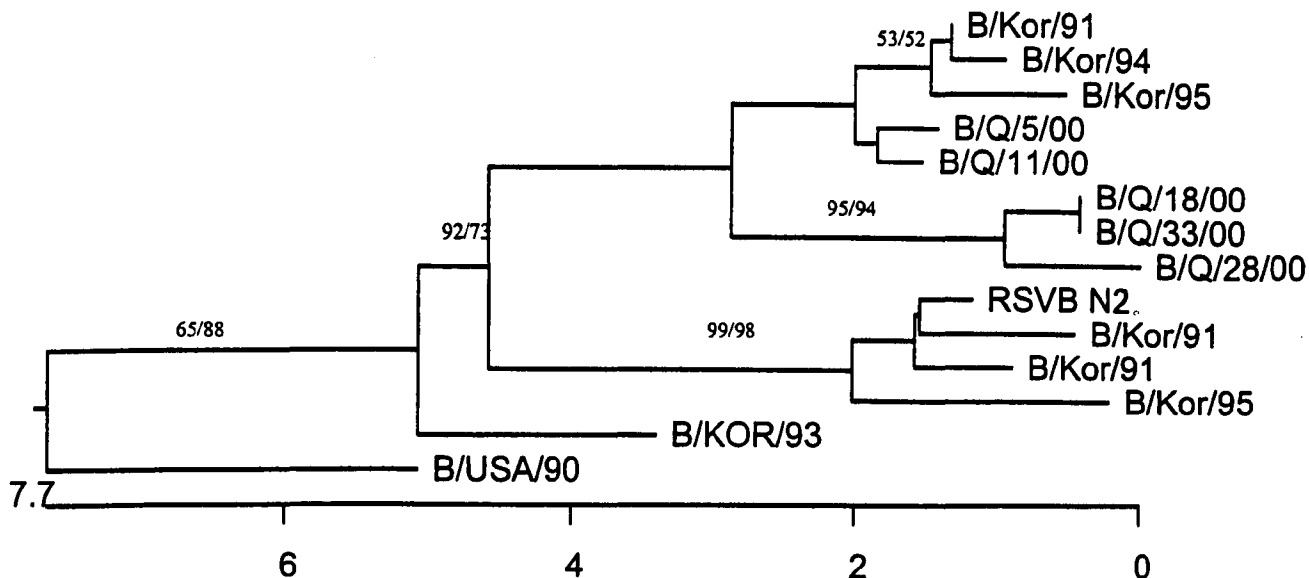
(b) Phylogenetic tree of the partial sequence of the RSV B G protein gene of Qatar & other world wide isolates encompassing amino acid 20 to 293 were include in analysis. Numbers in italic represent bootstrap proportions values (Distance/Parsimony method).

The sequences from Qatar RSV A isolates were closely related, for example in preliminary phylogenetic analysis using nucleotide sequences, appeared as a cluster showing A/Q/14/00 approximately 98.2% different from the nearest world wide isolate (A/K/98) while A/Q/10/00 had 15.3% similarity to the most distant isolate (A/USA/82). Using the sequence results from Qatar RSV B isolates for instance B/Q/5/00 was more closely related to the B/Kor/91 strain than it was to B/USA/90isolates, preliminary phylogenetic analysis using nucleotide sequences appeared as a cluster showing approximately 95.6 % identity & 9.3% divergence from the nearest Korean and the most distant USA isolates, respectively.

To accurately define the extent of genetic variability within and between groups, the amino acid sequence of the C-terminal end of the G protein gene was determined. The sequence determined corresponds to amino acids 192 to 298 of the A2 prototype strain and to amino acids 184 to 299 of the B8/60 prototype strain (**Figure 4. 7 a & b**). The deduced amino acid sequence of the Qatar group A isolates indicated four clusters of viruses A-1, 2, 3 & 4 with similar amino acid sequences(**Appendix 6**: Accession numbers for sequences that have been derived from databases)

Figure 4.7:(a & b): The phylogenetic tree, Comparison between the C- terminal sequence of Qatar and world wide isolates (July/2004)
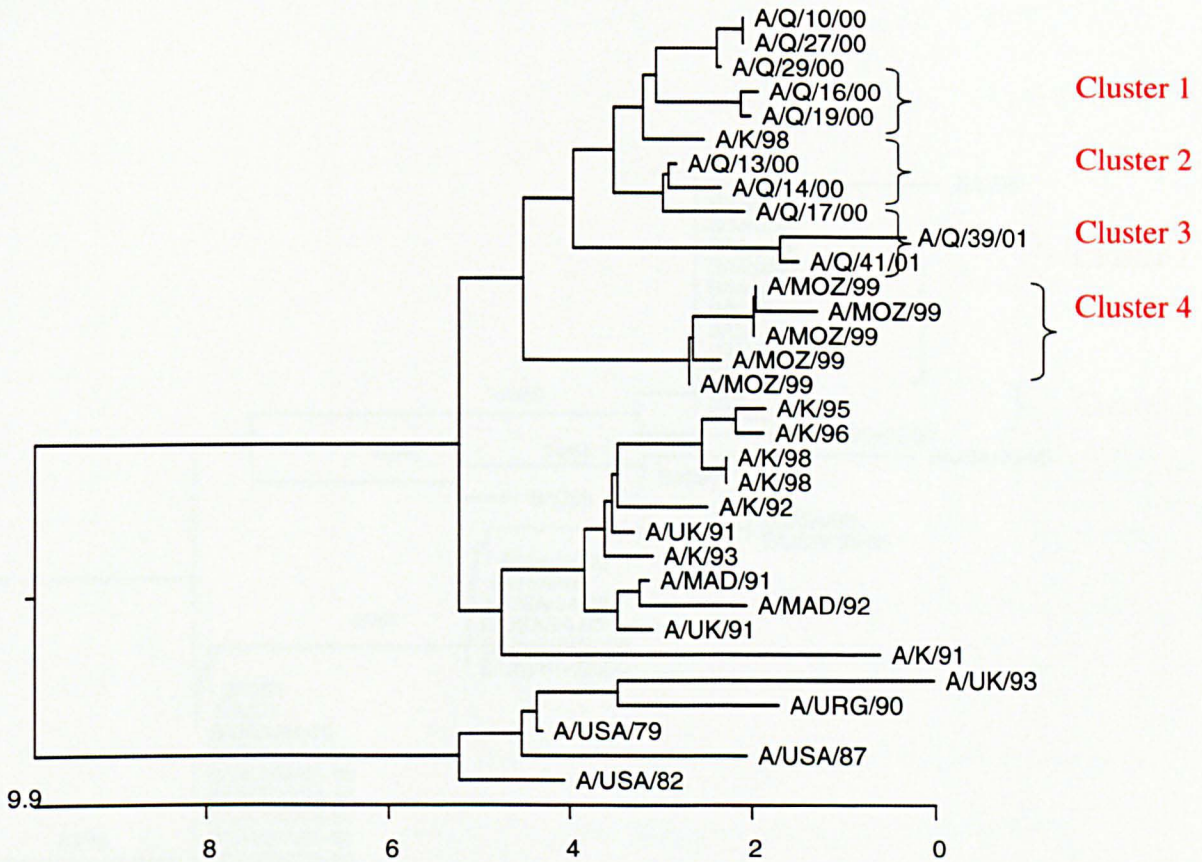
(a) Phylogenetic tree of the C-terminal sequence of the RSVA G protein gene of Qatar and other worldwide isolates, encompassing amino acids 192 to 296 in the analysis.

Figure 4.7:(a & b): The phylogenetic tree, Comparison between the C- terminal sequence of Qatar and world wide isolates (July/2004)
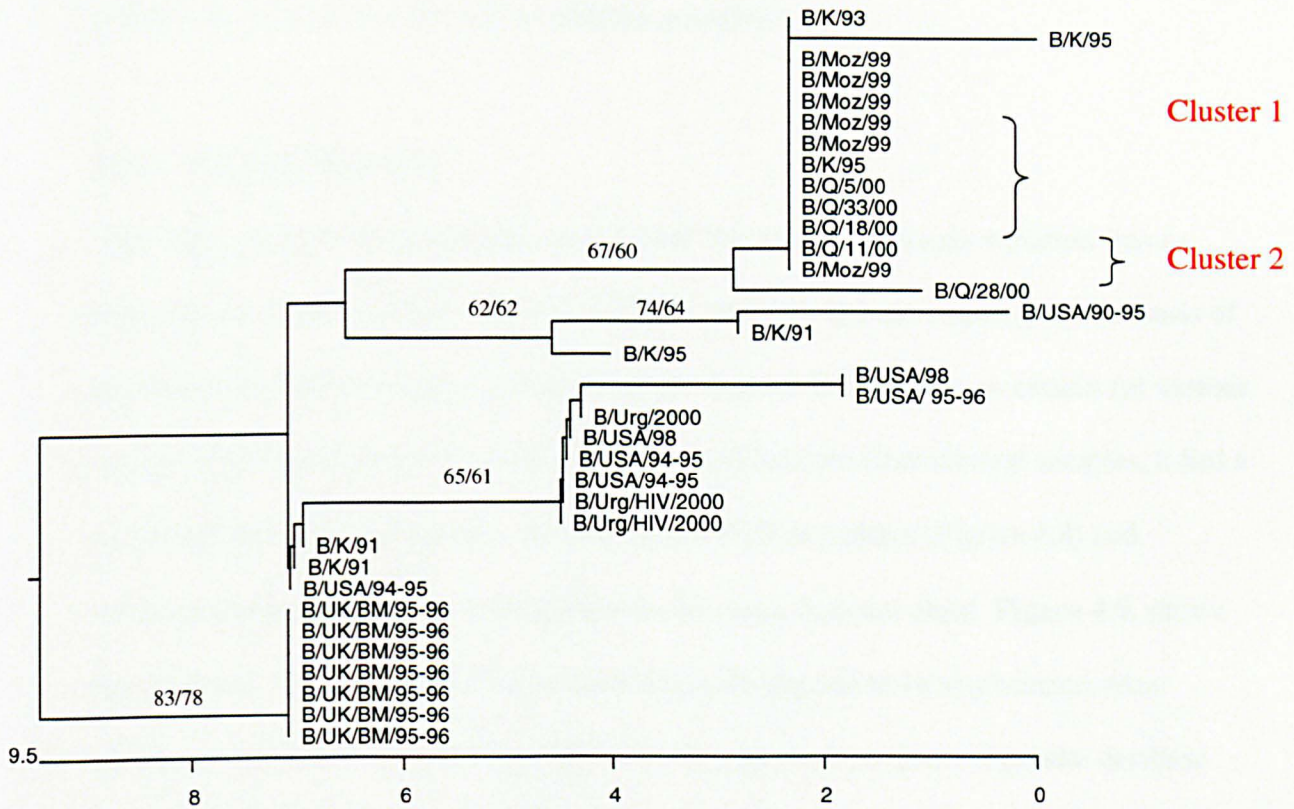
(b) Phylogenetic tree of the C-terminal sequence of the RSVB G protein gene of Qatar other worldwide isolates encompassing amino acid 184 to 293 were include in analysis.

The divergence is almost the same even at the carboxyl-end of the RSV B strain between Qatar strains and elsewhere was higher (2.3%) than in partial phylogeny (0.8%). In contrast to what was observed among Qatar B isolates where they were clustered together 67/60% of the time as assessed bootstrapping analysis.

4.2.4 B/Q/28/00 isolate:

The main target of the molecular work beside the analysis of strain variation was to select the most representative RSV B strains to perform epitope mapping by synthesis of peptides identified by sequence analysis of the G gene. B/Q/28/00 was chosen for various reasons. First of all, it was successfully grown and isolated from clinical samples, it had a longer nucleotide sequence than the other Qatar RSV B isolates (**Figure 4.8**) and secondly there were three sequential sera for the same infected child. **Figure 4.9.** shows the potential N and O -glycosylation sites along the peptide to be synthesized, from amino acid position 150 to 299, predicted by the ppsearch program of prosite database from EMBL (European Bioinformatics Institute). There are 25 potential O-glycosylation motifs and only two potential N-glycosylation motifs. Most of these sites are conserved among strains, especially the N-glycosylation sites, while O-glycosylation sites have some amino acid variation (mainly substitutions) compared with the amino acid sequences of other Qatar RSV B strains.

Extra computer analysis was done for the whole G protein of B/Q/28/00 to predict the structural characteristics of this fragment. Using the Deléage & Roux modification of the Nishkawa & Ooi 1987 program and the DNASTAR database it was found (**Table 4.3.** & **Figure 4.10**) from the predicted structural class of the whole B/Q/28/00 G protein that the most hydrophilic and antigenic part of this protein is located from amino acid position 150 to the C-terminal end.

Figure 4.8 : C-terminal end of the G gene of Qatar RSV B isolates

a

```
                        890           900           910
BN2       : CAAATTCCACCCAAAATACCCAATCACATGCTTAGTT

B/Q/5/00  : ................AC..T.G...T..........--

B/Q/11/00 : ................AC..T.G...------------

B/Q/18/00 : ................CC..T.G-------------

B/Q/28/00 : T...............C...........G..A......

B/Q/33/00 : ................C...........G..A......
```

b

```
              250       260       270       280       290       299
BN2       : ERDTSTSQSTVLDTTTLEHTIQQQSLHSTTPENTPNSTQTPTASEPSTSNSTQNTQSHA
B/Q/5/00  : .G.............AS...V.........................KP----
B/Q/11/00 : .G.............AS...V.........................KP----
B/Q/18/00 : ..............AS...V..........................P----
B/Q/28/00 : ..............AS...V.....................L........RD
B/Q/33/00 : ..............AS...V.............................RD
```

(a & b): C-terminal end of the G gene of Qatar RSV B isolates indicating two stop codon (TAG) positions that lead to two nucleotides (**a:** 896 & 900) and protein lengths (**b:** 295 and 299 amino acids). This result was obtained from a single sequence reaction with forward and reverse primer, which gave the same result.

Figure 4. 8 : C-terminal end of the G gene of Qatar RSV B isolates

c

+ a.a length	290	291	292	293	294	295	296	297	298	299
RSVB N2	N	S	T	Q	N	T	Q	S	H	A
B/Q/5/00, B/Q/11/00 and B/Q/18/00	N	S	T	Q	K	*P				
B/Q/28/00 and B/Q/33/00	N	S	T	Q	N	T	Q	S	R	D
Korea strain	S	T	S	N	S	T				
Mozambique strain 1	S	N	S	T	Q	K				
Mozambique strain 2	S	N	S	T	Q	K	L	Q	S	Y
USA strain	N	S	T	Q	K	A				
Japan strain	N	S	T	Q	K	L				
South Africa strain 1	N	S	T	Q	N	T	Q	S	R	A
South Africa strain 2	N	S	T	Q	K	L				
South Africa strain 3	N	S	T							

+ a.a : amino acid
*Termination codon. **E**: Glutamic acid, **P**: Proline, **S**: Serine, **N**: Asparagine, **T**: Theronin, **W**: Tryptophan, **R**: Arginine, , **K**: Lysine, **A**: Alanine, **L**: Leucine, **Q**: Glutamine, **Y**: Tyrosine and **D**: Aspartic acid.
Comparison between the G protein length of Qatar RSV B strains and elsewhere.

Figure 4. ٩: O & N glycosylation sites among G-protein of B/Q/28/00

B/Q/28/00

Red: Suggested peptide to be synthesised, from amino acid 150 to 299 position (Conserved and C-terminal regions).

Blue: Potential O-glycosylation sites, Predicted with confidence by NetOGlyc 2.0). (Between brakets): Potential N-glycosylation sites, Predicted by ppsearch program of prosite database from EMBL (European Bioinformatics Institute)

MSKHKNQRTARTLEKTWDTLNHLIVISSCLYKLNLKSIAQI

ALSVLAMIISTSLI

IAAIIFIISANHKVTLTTVTVQTIKNHTEKNITTYLTQVSPERV

SPSKQPTTTPPIHTNSATISPNTKSETHHTTAQTKGRTTTPTQ

NNKPSTKPRPKSPPKKPKDDYHFEVFNFVPCSICGNNQLCK

SICKTIPSNKPKKKPTIKPTKKPTIKTTKKRDPKTPAKTLKKE

TTTNPTEEPTPKTKERDTSTSQSTVLDTTASEHTVQQQSLHS

TTPENTP(NSTQ)TPTASEPSTL(NSTQ)NTQSRD

Figure 4.1o: Structural character of B/Q/28/00 predicted with protein programme of DNASTAR database.
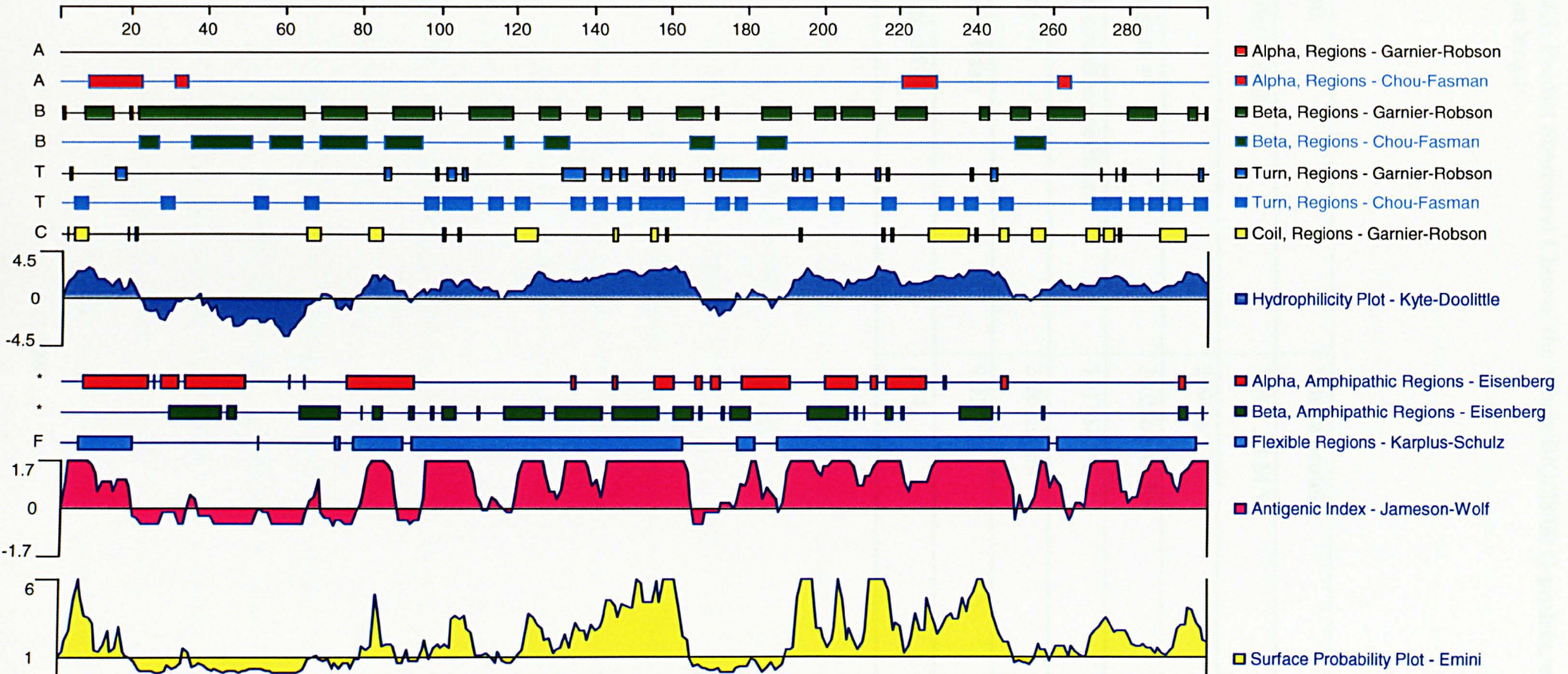
The most antigenic region among this protein localized between 150 – 290 amino acid positions.

Table 4.3: Predict Structural Class of the Whole B/Q/28/00 G protein, with expected length.

Analysis	Whole protein
Molecular Weight	33051.53 M.W.
Length	299 aa
1 microgram =	30.256 pMoles
Molar Extinction coefficient	1013+/-5%
1 A(280) =	3.26 mg/ml
Isoelectric Point	9.97
Charge at pH 7	25.42

4.3 Discussion:

Shifts in the predominant genetic group, circulation of multiple lineages and emergence of new lineages have been observed in several studies of human RSV molecular epidemiology (Cane & Pringle, 1995b).

In the work described in this study, primers were designed to amplify the whole G gene of RSV subtypes A & B. Analysis of this gene allows the differentiation and confirmation of isolates into strains and allows comparison of circulating strains for the study period. There are other published reports of primers that amplify parts of the RSV G gene (Sullender, 1993; Peret, 2000), but there is no publication with a complete set of primers for the entire G gene. The RSV G gene is highly variable and therefore it is necessary to ensure that the designed primers are appropriate for the strains being studied. In addition, most of the published protocols require a high copy number, good quality samples of cultured virus material and there are no reports of complete sequencing directly from clinical material as attempted here. Therefore, although some of the previously published primers were tested they didn't work well. The possible reasons for failure include; firstly the sensitivity of these primers was not optimised or secondly that the Qatar nucleotide sequence is different from nucleotide sequence of these primers. To overcome this, primers were designed to amplify the whole G gene, which were capable of detecting virus from clinical samples or tissue samples with equal efficiency.

The sequencing strategies designed were used initially to sequence an RSV A positive control (the study reference strain A2).

Samples which were identified as RSV positive from the study period were sequenced, depending on availability of serum and virus isolate. It was clear, however, from initial amplification reactions that there was a lower positive rate than expected as the samples had been through at least one cycle of thawing and re-freezing before being defrosted for PCR sequencing. To avoid this problem in the

second season, samples were prepared and stored as pellets to be ready for sequencing and some of them were stored as cDNA or PCR primary product, which are both more stable than non processed clinical material. Also, to increase the positivity rate, the samples should have been put into tissue culture immediately, which would increase the yield of virus and hence provide a higher copy number but a tissue culture facility wasn't available at HMC. However, the study was designed to produce a sequencing strategy that could be used directly with clinical samples and to perform epitope mapping by synthesising peptides of one of the RSV B G protein sequences obtained during this work.

It was apparent that the RSV G primers did not amplify as many samples as the multiplex primers (**Figure 4.3**), although the RSV G primers were shown to be as sensitive as the multiplex RSV primers on some isolates. It is possible that some of the currently circulating strains differ in sequence from those deposited in the Genebank Sequence Database from which the sequences were obtained for primer design (RSV A2 strain, isolated 1968, assigned accession number AF035006, (Whitehead et al., 1998). Also, the sequences deposited vary geographically and temporally. The other problem with sequencing is that the read-throughs for each sequence were only approximately 400bp so although primers were placed in the conserved region they were not always able to span across the whole of the variable region. One strategy to counteract this problem would be to use more primers in the sequencing reaction so that the forward and reverse primers overlap completely even if a shorter sequence is produced. In addition it could be that the viral load was lower in some samples for G primers.

Another explanation for the differences in detection of the RSV between the multiplex primers and the RSV G primers is differential expression of RSV genes. For non-segmented negative strand RNA viruses, it has been shown that the amount of mRNA that is transcribed depends upon the proximity of the genes to the 3' end of the

genome (Wertz et al., 1998). The multiplex primers are located in the N gene close to the genome 3' end, whereas the G gene is distant from the 3' end. It has been shown that a 15 fold decrease in gene product occurs when amplifying a gene further from the 3' end (Wertz et al., 1998). It is not certain whether mRNA or viral RNA (vRNA) was detected using this methodology, but it was believed that both were detected. However, if it was predominantly mRNA being detected it could explain the differences in detection sensitivity between the two methods. Also, Fearns et al., (Fearns et al., 2002) indicate that for RSV, the transcription promoter lies at the very 3' end of the genome, irrespective of the location of the site of transcription initiation, and overlaps with the replication promoter. Among the first 11 positions there was flexibility at position 4: the assignment of C, G, or U at this position was compatible with a high level of RNA synthesis, but the optimal assignment was different for transcription versus replication. A G residue was associated with a lower level of replication and a higher level of transcription than either a U or a C residue.

The final primer set or strategy was tested on the clinical isolates obtained from Qatar. Seventeen RSV A samples and nine RSV B samples were amplified using the nested reaction with primers SH136F and F1R. The methods had to be modified when testing clinical material, which were of poor quality because with the original cycling conditions it was not possible to amplify the G gene. However, once the number of cycles was increased and the annealing temperature was lowered, more samples were amplified. Although amplification was successful, some bands were smeary and the correct size band was not easy to identify using long wave light. Therefore, the cycling conditions should be re-optimized using clinical material which has a lower copy number than control material. However, because of time limitations only twenty-six isolates, which produced a strong signal, were chosen for sequencing. When the sequencing strategy was employed complete sequences were obtained for fifteen isolates.

After success with amplification of test clinical isolates, samples that were identified as RSV positive in children for whom there were blood samples (the Qatar study children) were used to attempt direct amplification and sequencing. It was clear, however, that it would be difficult to amplify the samples and obtain sequence information from all the Qatar study children. Difficulty in amplification and sequencing of G protein gene from clinical material may be overcome by re-designing the primer. Another possibility would be to further optimise the conditions for amplification including varying the concentration of Taq. In order to improve the methodology, a proof reading enzyme should be used to reduce errors. A further method that could be used is cloning of the selected fragment, which enables easier sequencing, improves accuracy and the reduction of quasispecies, which could result in poor quality of sequences. Cloning was not used in this study because it was not certain that amplification of the whole of the G gene would be possible. Also it is an expensive method in materials and it is labour intensive. And it is not certain whether the sequences obtained are representative of the majority species in the sample.

An identical G gene sequence was found when comparing sequences derived directly from the original NPA samples and from RSV isolates derived from the same material passed in Hep 2 cells. Lack of host selection has been described elsewhere (Zambon et al., 2001; Garcia et al., 1994).

Genetic variability was analyzed for RSV isolates and viruses were characterized to antigenic group (group A or B) by both RT-PCR and GPCR, although viruses of both antigenic groups were present each year, the group A viruses were dominant for both years. Similar variability has been described in other studies, and overall, the group A viruses are more frequently identified than the group B viruses (Coggins et al., 1998; Anderson et al., 1991; Cane, 1997; Cane et al., 1994; Hendry et al., 1989). In a study performed in Finland, alternating group A and B virus dominance has been observed

(Waris, 1991), while in two studies performed in India and Mozambique group B virus was dominant for two seasons (Rajala et al., 2003; Roca et al., 2001).

In addition to the differences in the viral groups isolated each year, sequence analysis showed that the group A viruses which were dominant during the two seasons were genetically distinct (2 clusters). Previous studies suggested that there was less variability among group B viruses (0.8%) than among the group A viruses (5%) (Coggins et al., 1998; Sullender et al., 1991). The data presented here confirm this observation for isolates from a period spanning two epidemics at a single location. However, the group B viruses have the potential for additional variability as evidenced, by comparisons to prototype and between Qatar isolates more (13%) than between Qatar isolates and elsewhere (10%) especially at the nucleotide level. Whether the analysis of additional group B isolates would reveal even greater differences remains to be determined especially with a limited number of complete sequences available in the GenBank. The extent to which the individual viruses vary locally and over time could influence the pattern of reinfections with different viral strains. The group A viruses may vary more extensively than the group B viruses, this might play a role in the studies of RSV epidemiology.

While three different lengths (292, 295 and 299 amino acid) of the G protein gene have been described among B group viruses (Garcia et al., 1994; Roca et al., 2001), 3 lengths also (295, 298 and 299 amino acid) have been found among group A isolates (Garcia et al., 1994). In the current study, all Qatar group A sequences had a G protein lengths of 297 or 298 amino acids, and two G protein lengths were observed among group B strains (2 isolates 299 amino acids and 3 isolates 295 amino acids). These results were obtained from a single sequence reaction with a primer set but the same result was given with the forward and reverse primer. Changes in stop codon usage have been associated with important antigenic variations found in RSV escape mutants that were selected with monoclonal antibodies recognizing strain-specific

epitopes (Melero et al., 1997). In addition, assessment of the length of the protein G could have immunogenic implications in an RSV outbreak.

Although a single experiment indicated longer sequences, this phenomenon has been observed elsewhere and consistent with other work (Roca et al., 2001; Venter et al. 2001). Nucleotides addition was not a single point mutation, insertional mutation, duplication or read-through error but it may have arisen by a more complicated mechanism, perhaps due to a polymerase error, leading to addition of multiple non-templated nucleotides.

Others have reported this phenomenon of premature termination (by adding and removing last amino acids from viral genome due to insertion or deletion mutation) and its affect on the antigenicity of the protein, with other viruses. For instance in HIV, antigenic variation in multiple epitopes can lead to complicated antigenic oscillations, especially in CTL responses against multiple epitopes located on the terminal end of HIV genome (the NH2-terminal flanking region) (Nowak et al., 1995a; Nowak et al., 1995b; Draenert et al., 2004).

Since there is less information about group B sequences (the complete genome) than about group A sequences, further analysis of group isolates of different geographical origin will help to clarify the uniqueness of viruses from group B. Our data obtained here confirm that strains of RSV circulating in a single season may be genetically more closely related to strains isolated in very distant places in different years than to co-circulating strains of the same group isolated during the same outbreak (Melero et al., 1997). Hence, these data reinforce the high capacity of RSV for worldwide spread (Garcia et al., 1994).

Only sporadic amino acid changes were observed among sequences from the same cluster and most of them correspond to amino acids present in strains from the other cluster. These data suggest that at certain positions, and despite the sequence heterogeneity of RSV, the acceptance of specific amino acids was restricted. This fact

could have important implications in the design of broadly reactive synthetic vaccine candidates.

Different patterns of putative N- and O-glycosylation sites, which may play an important role in the mechanisms involved in virus escape from host immune pressure, have been previously described in group A viruses (Cane & Pringle, 1991). The present results indicate the presence of 4 N-glycosylation sites among the G protein of group B Qatar strains. Therefore, and in agreement with the lower sequence heterogeneity of group B isolates, our data suggest that the potential antigenic variability mediated by changes in the putative N- and O- glycosylation pattern of group B isolates is different from that of group A isolates (7 N-glycosylation sites). Whether these different patterns also have implications in the higher antigenic variability detected among group A isolates remains to be established. Different RSV virus strategies to escape from the immune response have been studied, (Palomo et al., 2000) and it has been suggested that the contribution of carbohydrates to the antigenic structure of the G protein C-terminal third is of crucial importance, because this influences the expression of certain epitopes, either masking the epitope or contributing to antibody recognition.

Phylogenetic analysis showed that the group A and group B viruses were placed in multiple lineages. Most of the major phylogenetic branches included viruses, which were isolated in this study, reflecting the great diversity of RS viruses, which may be present in a community over a period of 2 years. However, among the group A viruses it was also clear that viruses isolated at different times and from different places could be very similar to the viruses isolated from the Qatar study children. Thus, viruses from Spain and Korea, which were isolated several years before the viruses studied here, could be grouped phylogenetically with these viruses. The observation that very similar viruses are isolated at different times and from geographically distant sites

demonstrates that the virus is capable of worldwide spread (Melero, 1997; Muelenaer, 1991).

The study presented here was designed to assess variability in a detailed manner over a limited period of time. Thus, the study was not intended to address the issue of change over time. For the group A RS viruses, there appears to be an accumulation of change over time (Melero et al., 1997). This observation is also compatible with accumulation of changes among the group B RS viruses. However, until a greater number of more chronologically and geographically disperse isolates have been examined, this issue remains speculative for the complete genome of the G protein for the group B RS viruses.

The data also shows that variability occurs among group B RSV viruses (0.8%), although to a lesser extent than among the group A viruses (5%). These data indicate that the group B RS virus G proteins, while less variable in this study, are no more likely to have mutations than are the group A RS virus G proteins. The high percentage of nucleotide changes which resulted in amino acid coding changes (Cane, 1991; Sullender, 1991) . One possible advantage would be that such change results in an escape from the host immune response. This might contribute to the ability of RS viruses to establish infections throughout life. Also, genetic diversity permits RNA viruses to refine their adaptation to a constant environment, and to have a certain probability of finding adaptive routes in response to environmental change.

It would be interest to study the effect of losing or adding some amino acids to the G-protein of RSV (especially RSV B), by developing an assay to detect antibodies react with different RSV G-protein length either by cloning and expressing the different length ends of this protein (Jones et al., 2002) or by using different peptide length (glycosylated and non- glycosylated).

These results confirm the variability among group A RS virus isolates which has been demonstrated previously and the loss of some terminal amino acids (especially in

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RSV B strain) (Coggins, 1998 ; Cane, 1995 ; Garcia, 1994) . Higher abundance of

nonsynonymous (amino acid altering) mutations versus synonymous (silent)

mutations was found in the C-terminal end and there are several reports that show the

advantages of nonsynonymous substitutions during RNA virus evolution. One

example is the variable secondary structural domain at the internal ribosome entry site

of picornaviruses (Pilipenko et al., 1989). There is evidence that secondary structure

constraints have imposed limitations for the evolution of RNA genetic elements

(Yamamoto & Yoshikura, 1986). Thus, mutations that alter such higher-order

structure- irrespective of their effect on the first, second, or third base position in

codons- must be subjected to negative-selection. Structural and regulatory elements

within the genomic RNA are as much a part of the phenotype as are viral protein-

encoding segments. Negative-selection may act to eliminate synonymous as well as

non-synonymous substitutions. The dominant targets of negative-selection (RNA or

protein) may depend on the environmental condition being imposed on virus

replication (type of cell, type of infection-acute or persistent, modulate by other

RNAs- access of virus to antibodies and to other inhibitory molecules and so on. Such

an evolutionary phenomenon in the absence of immuno-selection pressure, as with

Ross River virus in a nonimmune population, was associated with a surprisingly low

evolutionary rate of the virus (Burness et al., 1988). Clearly, the most powerful

potential force directing viral evolution is the host's immunological response. Yet in

the laboratory, neutralization escape mutants can readily be found for all viruses.

Therefore, the paradox of high viral mutation rates and (usually) high antigenic

stability in vivo requires explanation. In the case of influenza, the most notable

example of viral antigenic instability, the infection is usually initiated by only a few

infectious units (Alford et al., 1966), is short-lived, confined to the respiratory

epithelium, and is characterized by subsequent imperfect immunity. Therefore, in

primary infection, little opportunity for immunoselection from a large initial viral

population exists. Rather, such selection most effectively occurs with reinfection of, and passage in, partially immune hosts. These events lead to a self-propagating sequence of pervasive epidemics, leading to widespread partial immunity, followed by emergence and preponderance of viral populations best equipped to survive. In those infections, respiratory or otherwise, in which such viral immunoselection is not usually evident in nature (e.g. measles), the opposite conditions obtain; that is, infection is relatively protected, involves multiple viscera, and engenders more solid immunity, so that the opportunity for virus to flourish in the presence of antibody during subsequent challenge is reduced or absent as reinfection is diminished (Luoh et al., 1992).

5 Chapter 5: Analysis of specificity of anti-RSV sera using synthetic peptides from the G protein of RSV B

5.1 Introduction:

The use of vaccines is the most effective means of preventing infectious diseases. **Table 5.1** summarizes the development of human vaccines. Besides the high cost, conventional vaccines have several disadvantages including lack of stability and requirement for refrigerated storage, which is difficult to maintain in tropical and developing countries (Pichichero et al., 1990). Above all, conventional vaccines require great attention in their preparation to avoid the risk of infection, which may arise with attenuated vaccines reverting to virulence. On the other hand, inactivated vaccines must be totally innocuous (Francis, 1990; Arnon & Sela, 1985; Olszewska et al., 2004; Polack & Karron, 2004). For these reasons scientists have diverted their attention towards the development of alternative vaccine approaches. The fact that HBsAg (the surface antigen of hepatitis B virus), which is found in excess in the plasma of infected individuals, can protect against the whole virus particle has focused attention on the potential of subunit vaccines (Francis et al., 1986). By understanding the nature of the antigen-antibody interactions involved and which part of the virus or the microorganism is required to provide full protection, a vaccine can be designed from artificially engineered virus particles, viral subunits, isolated proteins or synthetic peptides. This can be achieved by identifying those sites involved in stimulating a protective immune response. Having obtained this information, it is then possible to design a potential vaccine, which contains only the important region for the induction of protective immunity. Therefore, studying the antigenicity and immunogenicity of a protein or antigen has become a major scientific activity with an increasing emphasis on the potential use of selected viral proteins as the basis of vaccines against a variety of diseases.

Table 5.1: Outline of the development of human vaccines.Adapted from Plotkin and Orenstein, 1999

Live Attenuated	Killed Whole Organism	Purified Protein or polysaccharide	Genetically Engineered
Eighteenth Century			
Smallpox, 1798			
Nineteenth Century			
Rabies, 1885	Typhoid, 1896		
	Cholera, 1896		
	Plague, 1897		
Early Twentieth Century	Pertussis, 1926	Diphtheria, 1923	
Tuberculosis, 1927	Influenza, 1936	Tetanus, 1927	
Yellow fever, 1935	Rickettsia, 1938		
After World War II			
(Cell Culture)	Polio (injected)	Pneumococcus	Hepatitis B
Polio (oral)	Rabies (new)	Meningococcus	recombinant
Measles	Japanese encephalitis	H. influenzae PRP	(Yeast- or
Mumps	Hepatitis A	Hepatitis B (plasma derived)	mammalian
Rubella		Tick-borne encephalitis	cell-derived)
Adenovirus		H. Influenza PRP-protein (conjugated)	
Typhoid (Salmonella Ty21a)		Typhoid-Vi	
Varicella		Acellular pertussis	

PRP, Phosphosylribitol phosphate.

The term antigen refers to any entity that can be specifically recognized by antibody molecules and the cellular components of the immune system. The antigenic determinant (epitope) corresponds to those parts of the antigen that are specifically recognized by the binding sites (paratopes) of immunoglobulin molecules (Van Regenmortel, 1989). Thus, epitopes are those entities that require a complementary paratope for their operational recognition and therefore the particular antibody becomes known as an antibody specific for that antigen. Antibody molecules contain two identical paratopes made up of six highly accessible loops of hypervariable sequence known as complementarity determining regions (CDRs) capable of forming an enormous amount of sequence variation in order to bind to myriad different antigens. The identity of an immunoglobulin is thus established after its complementary antigen has been identified. It has been postulated that the number of epitopes in a protein can be estimated from the total number of different mAbs that it is able to induce (Amit et al., 1986).

Epitope types: The antibody response to a protein is predominantly focused to restricted parts of protein molecules termed B-cell epitopes, that are made up of amino acids. Epitopes are divided on the basis of their molecular structure into two main categories, sequential or continuous epitopes and conformational or discontinuous epitopes. The former is defined as a short linear stretch of residues which are located in the primary polypeptide sequence, whereas discontinuous epitopes consist of a group of residues that are not contiguous in the primary sequence but are brought together by the folding of the polypeptide chain. In general, a linear peptide fragment of a protein reacts less readily with antibodies raised against the intact protein than does the intact native antigen itself. This is partly because of the inability of the peptides to adopt the correct conformation, and secondly, the linear peptide may represent only a part of a larger discontinuous epitope and reacts only with antibodies directed to this epitope.

Not every residue in a linear epitope is necessarily a contact residue with the paratope (Geysen et al., 1988) as some of the residues in a continuous epitope can be replaced by any of the other 19 amino acids without impairing antigenicity. It is now widely believed that most of protein epitopes are conformational and very few linear stretches of residues are present at the surface of a protein (Leach, 1983; Chargelegue et al., 1997). However, most studies aimed at revealing protein antigenicity have focused on the cross reactivity between proteins and short linear peptides (Van Regenmortel et al., 1978).

There are also three other types of epitopes, e.g.; first group of epitopes, which are hidden and become exposed only after fragmentation or denaturation of the antigen (Van Regenmortel, 1992). Second group of epitopes that are specific for the quaternary structure of proteins; they arise as a result of conformational changes induced by intersubunit bonds of residues from neighboring subunits, and are commonly found in viral capsids (Van Regenmortel, 1966). Third group of epitopes (neutralization epitopes) correspond to those epitopes of infectious agents and toxins that are specifically recognized by antibody able to neutralize the biological activity of the antigen. The identification of neutralization epitopes is important for the development of synthetic vaccines because it is this type of epitope that should be mimicked by synthetic peptides and used for eliciting protective immunity.

Antigenicity of RSV: Two antigenic subgroups of RSV, designated A and B, have been distinguished on the basis of the two antigenic and sequence dimorphisms that are most pronounced for the ectodomain of the G protein (as previously described in the Introduction), one of the two major protective antigens. Although there is substantial cross-reactivity between the two subgroups, they are sufficiently distinct that an effective RSV B-cell epitope based-vaccine will need to protect against infection with both. Thus a potential B- cell epitope-based vaccine to RSV should

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contain B-cell epitopes from both A and B subtypes of G protein. Most studies have been conducted with B-cell epitopes from RSV A isolates (**Appendix 7**), because sequence data for the G protein of subgroup B RSV strains are much more limited than are those of subgroup A. In addition, most work on RSV A has been performed with laboratory - adapted strains. Finally because most RSV B studies are widely separated, either geographically or chronologically, focus has been on RSV A. From the information in **Appendix 7**, it can be concluded that most of the work on B cell epitopes was focused on the conserved region of protein G. The exception was the work of (Sullender, 1990; Sullender, 1995) who used the whole G protein as expressed protein and in both their studies they used non-human sera. Only (Akerlind-Stopner et al., 1990) used peptides from part of the C-terminal end (including the conserved region) of the G-protein with human sera. However, it was not specified if the sera had neutralizing activity.

The rest of the studies have been done on the RSV A G protein and have identified different amino acid sequences recognized by either human sera or as protective epitopes (Norrby et al., 1987; Plotnicky-Gilquin et al., 1999; Power et al., 2001; Dagouassat et al., 2001). Antibodies to some of these epitopes were able to confer protection.

Methods used for epitope identification: The most commonly used methods to localise protein epitopes consist of identifying which fragments of the molecule are able to cross react with antibodies raised against the native protein. Several different strategies for identifying epitopes have been described. Some of these studies involved the use of the protein molecule itself such as X-ray crystallography as in the case of the neuraminidase of influenza virus (Colman et al., 1983). This method is based on structural analysis, since it is the only method that gives selective attention to the visual appearance of the spatial arrangements found in the antigen-antibody complex at a specific time. However, this method is labour intensive and requires

special expertise. Other approaches involve the use of fragmentation of the native protein, either by chemical cleavage or by controlled proteolysis. The resultant fragments are then screened for immunologically active domains that can bind to antibodies and interfere with their interaction with the intact antigen (Arnon & Sela, 1985). These methods are based on functional analysis taking into account the results of binding measurements. They have been used successfully for the identification of complete antigenic structures of myoglobulin, hen egg white lysozyme and an almost complete antigenic structure of serum albumin (Atassi, 1979). The method based on structural analysis cannot provide direct evidence that every residue contributes to the binding reaction. On the other hand, binding measurements are necessarily of an operational nature depending on the type of probe used for assessing immunochemical binding: for instance, free peptide versus conjugated peptide, short peptide versus a more constrained long peptide, liquid versus solid-phase immunoassay, monoclonal versus polyclonal antibodies. These methods actually analyse cross-reactivity instead of antigenic reactivity; and lead to the conclusion that a much smaller number of residues (1 to 5) are critical for antibody binding and thus that the epitope defined in a functional analysis involves fewer residues than may be defined in structural terms. The development of solid phase peptide synthesis and methods for the testing of large numbers of peptides on solid supports has provided new insights into the molecular basis for antibody specificity and the mechanisms by which the antigen and antibody combine. Recently, a number of alternative approaches have been also described that involve the systematic synthesis and screening of large peptide libraries of defined structure (Arnon & Sela, 1985).

The aim of this part of the work is to identify B-cell epitopes recognized by human sera obtained before and after infection using a panel of 15-mer overlapping synthetic peptides corresponding to the RSV G protein of a recent RSV B strain isolate.

A widely used approach to the identification of B-cell epitopes is the use of

overlapping synthetic peptides that span the entire sequence of a protein antigen

synthesized using the Fmoc procedure. This synthetic approach is much milder than

the other peptide synthesis chemistry, tBoc chemistry, because the peptide chain is not

subjected to acid conditions during each cycle and the final deprotection and cleavage

steps can be achieved using Trifluoroacetic acid (TFA) rather than the much stronger

hydrofluoric (HF) acid conditions (Arnon & Sela, 1985).

The size of the peptides chosen was 15 amino acids overlapping by five amino acids

because B cell epitopes range between 15- 22 amino acid, in length. It has been

reported that only three to five residues of the structural epitopes contribute

significantly to the binding energy i.e. 3-5 a.a. are in intimate contact with the

antibody and are therefore considered as critical amino acids; deletion of one of these

amino acids might render the peptide not reactive. Therefore it is advisable to

have the peptides overlapping by 5 amino acids. These peptides are then tested for

their capacity to serve as targets for B-cell epitopes in vitro.

In the work described in this chapter, 17 15-mer overlapping peptides (5 amino acid

overlap) corresponding to residues 150-299 of the G protein of RSV B strain

B/Q/28/00 isolate (**Figure 5.1 a+b**) were used (**Appendix 8:** amino acids

abbreviations). This sequence represents the conserved and C-terminal region and

avoids the trans-membrane region. This RSV isolate was chosen for this part of the

work because it had been isolated from a child for whom 3 sequential sera were

available and the post-infection serum had a high titre of neutralizing antibody

(Chapter 6). The (B/Q/28/00) isolate and sequence analysis was available for this

strain (Chapter 4) with longest C-terminal end (923 nucleotides). Melero et al (1997)

suggested that the C-terminal of the G molecule folds back in the three –dimensional

structure, so that the C-terminal end is in close proximity to the cysteine cluster and

he considered that some epitopes (**Figure 5.2**) which afford maximal neutralization,

would be in close proximity to the putative receptor binding site of the G molecule (all this work had been performed with the laboratory adapted strains of RSV). Three types of epitope have been identified in the G protein by mapping using escape mutants the reactivity of monoclonal antibodies with a large panel of viral isolates: i) conserved epitopes that are present in all RSV isolates; ii) group specific epitopes shared by all viruses of the same antigenic group, and iii) strain-specific or variable epitopes that are present in certain isolates of the same antigenic group (Melero et al., 1997). The strain-specific epitopes have been mapped within the hyper variable C-terminal third of the G protein ectodomain (**Figure 5.2**). It has been shown that the C-terminal region of the RSV G protein includes epitopes important for the neutralization of the virus and that loss of the terminal amino acids can allow escape from antibody neutralization (Cane et al., 1992).

For the work described here, it was hypothesized that maximum peptide recognition by human sera could be obtained with peptides from the conserved part of G protein and some from the C- terminal part of this protein of RSV B as had been found with RSV A. In addition, the possibility exists that the pattern of peptide recognition varies with the group of sera (children, adult and neonatal sera) and some of these sera could contain high affinity antibodies. By assessing the affinity (the tightness of antibody binding to the antigenic determinant) of antibodies of some sera that reacting with these peptides it may be possible to identify if this part of the protein contains protective epitopes that inducing high affinity antibodies. If so, then peptides from this region can be used to develop a potential vaccine. The objective of this work was to test these hypotheses.

Figure 5.1 (a&b): Peptide alignments

KPRPKSPPKK**PKDDY**HFEVF**NFVPC**SIC GN**NQLCK**SICK

15   14   13   12

T**I**PSNK PKKKP**TIKPT**KKPTI**KTTKK**RDPKT**PAKTL**KKE

16   17

11   10   9   8

TT**TNPTE**EPTPK**TKERD**TSTSQ**STVLD**TTASE**HTVQQ**QS

7   6   5   4

LHS**TTPEN**TPNST**QTPTA**SEPST**LNSTQ**NTQSRD

3   2   1
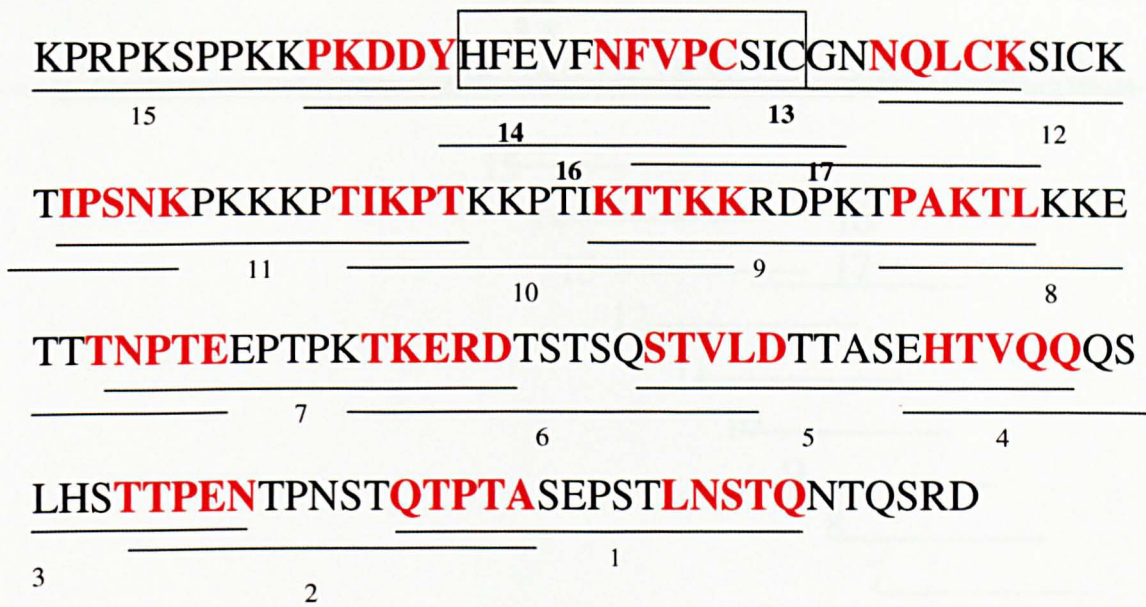
(a) Fifteen overlapping synthetic peptides (overlapping by 5 amino acid in red letters) representing amino acid sequence 149-299 of RSV G protein (B/Q/28/00). The C-terminal region with two variable regions that is separated by the most conserved region in both subtypes A&B (13 a.a in box) is included but the transmembrane region is excluded.

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Figure 5.1:(a&b): Peptide alignments

SH   **G**   F

15 14 (16 & 17) 13 12 11 10 9 8 7 6 5 4 3 2 1
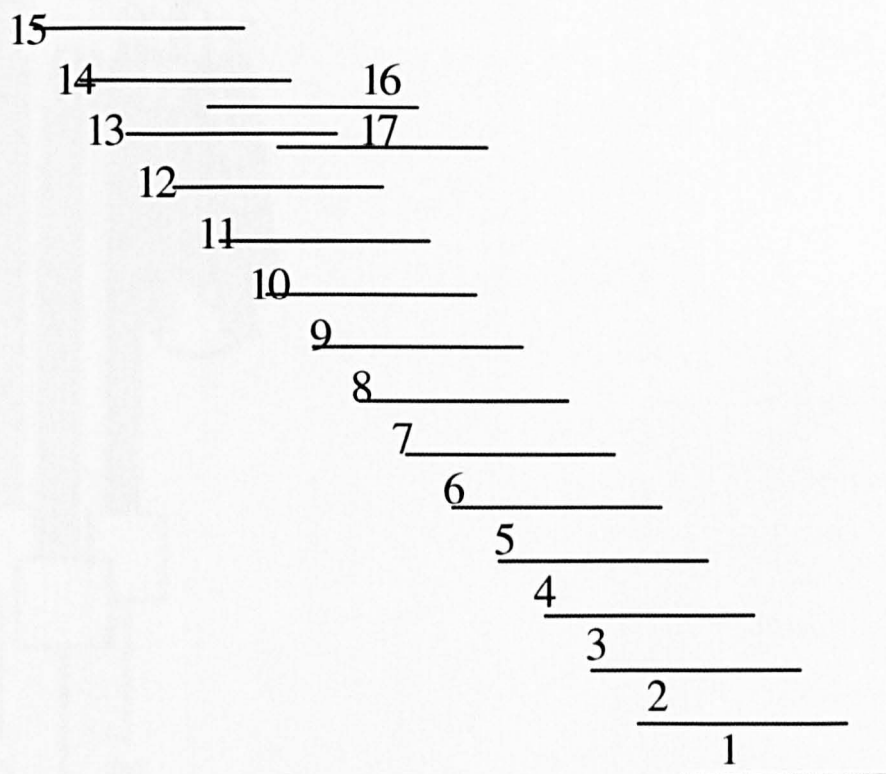
(b) The peptides are numbered 1 through 15 starting from the COOH-terminal end of the protein.

Figure 5.2: Model of the three dimensional structure of the G protein (Melero et al., 1997).
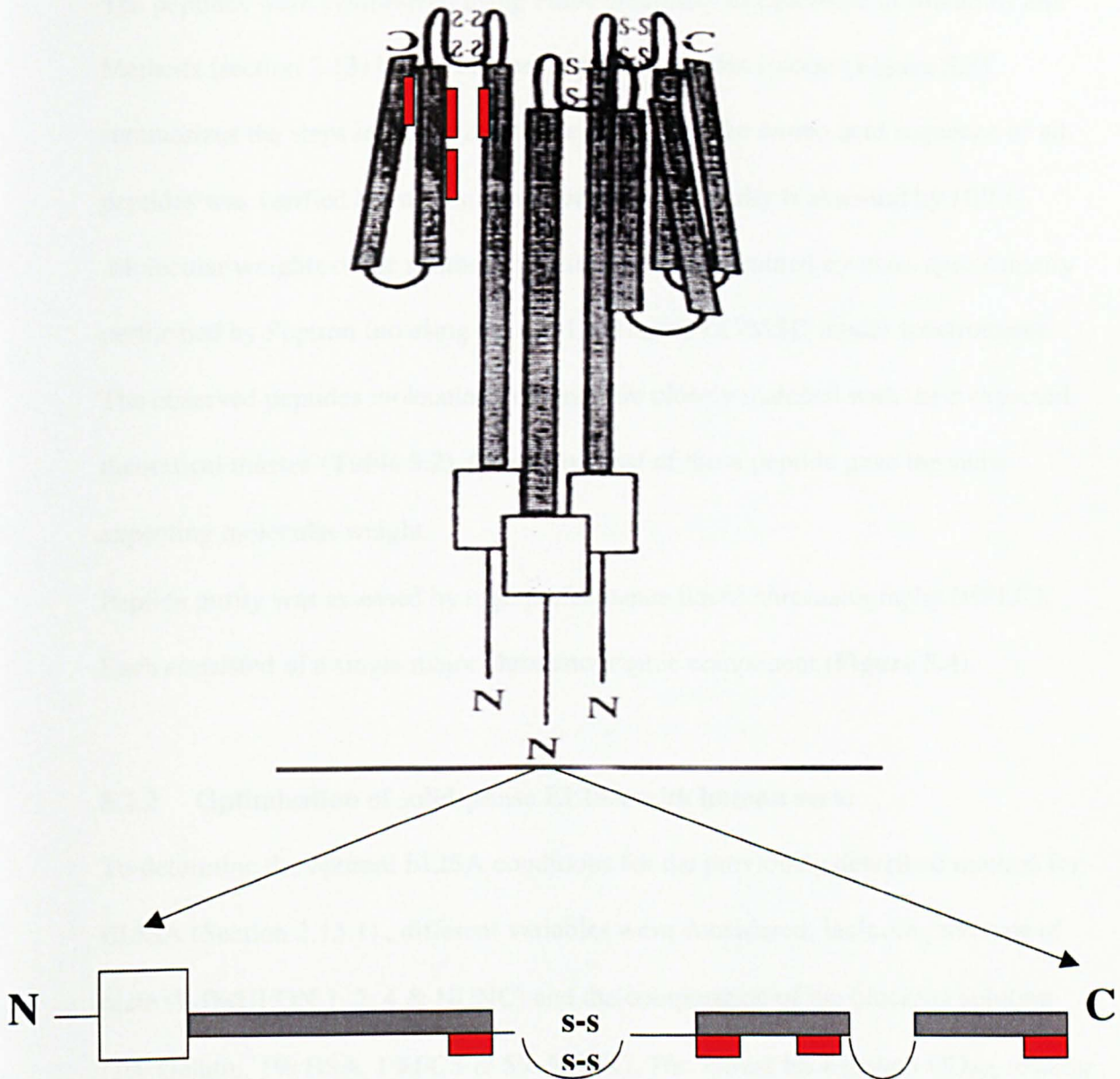
Disulphide bond (**S-S**) and the **N-** and **C-** terminal ends are indicated. White rectangles indicate the transmembrane domain and gray rectangles the variable regions. Red rectangles indicate the location of epitopes that induce maximal neutralization activity.

5.2 Results:

5.2.1 Peptide characterization:

The peptides were synthesized using Fmoc chemistry as described in Materials and

Methods (section 2.13) by the Pioneer peptide synthesis system (**Figure 5.3**).

summarizes the steps involved in peptide synthesis. The amino acid sequence of all

peptides was verified by mass spectrometry and the purity is assessed by HPLC.

Molecular weights of the synthetic peptides were determined by mass spectrometry

performed by Peptron Inc using the HP 1100 series LC/MSD model spectrometer.

The observed peptides molecular weights were closely matched with their expected

theoretical masses (**Table 5.2**). Generally most of these peptide gave the same

expecting molecular weight.

Peptide purity was assessed by high performance liquid chromatography (HPLC).

Each consisted of a single major chromatographic component (**Figure 5.4**).

5.2.2 Optimisation of solid-phase ELISA with human sera:

To determine the optimal ELISA conditions for the previously described method for

ELISA (Section 2.15.1) , different variables were considered, including the type of

plate (IMMULON 1, 2, 4 & NUNC) and the composition of the blocking solution

(1% Gelatin, 1% BSA, 1%FCS & 5% MILK). The lowest background OD_{492} reading

was achieved with IMMULON 4 plates using 1% BSA as blocking solution.

5.2.3 Samples

The following panels of sera were used to test their reactivity with peptides; (i) Qatar

study sera taken before and after infection; (ii) Qatar neonatal samples and (iii)

control sera from UK adults (**Table 5.3**)

Figure 5.3: Schematic diagram of peptide synthesis using Fmoc chemistry.
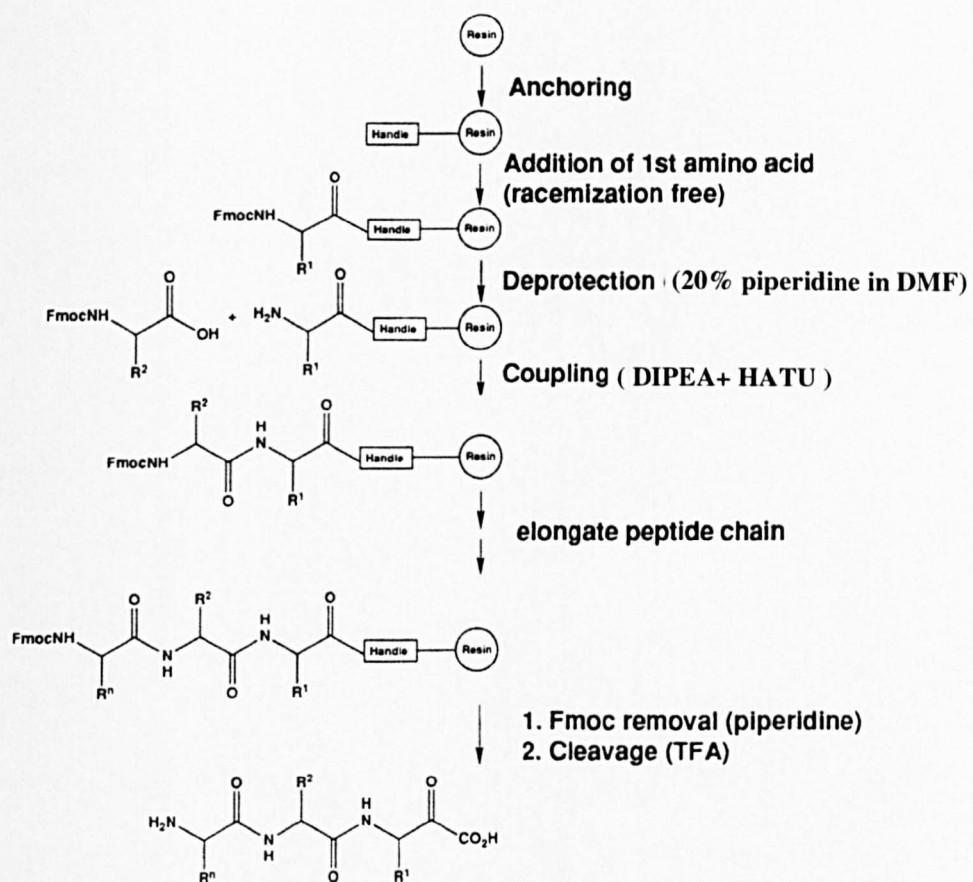
Table 5.2: Mass spectrometry analysis.

Peptide	Mass expected	Mass found
1	1262.58	1263
2	1560.73	1562
3	1558.71	1559
4	1705.8	1705
5	1615.77	1616
6	1666.8	1667
7	1741.85	1742
8	1657.89	1657
9	1712.03	1713
10	1712.09	1713
11	1676.03	1677
12	1675.87	1676
13	1638.73	1638
14	1855.82	1857
15	1780	1780
16	1760.77	1762
17	1808.84	1809

Generally most of this peptide gave the same expecting molecular weight.

Figure 5.4: Representative example of HPLC analysis.
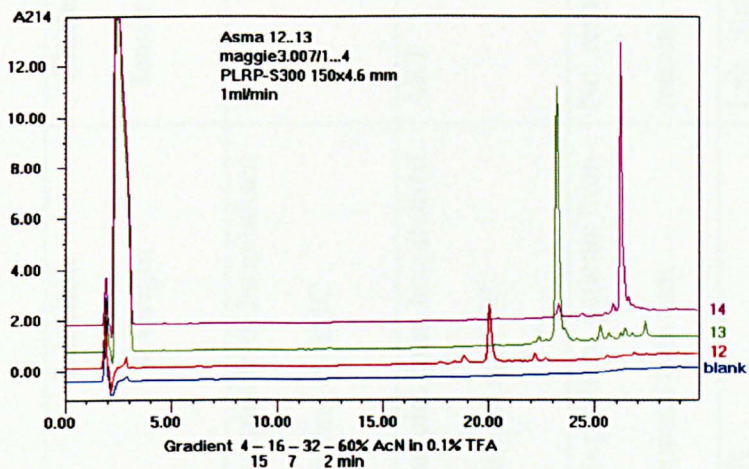
HPLC chromatographs of peptide 12, 13 & 14 HPLC are shown. The purity of peptides was: peptide 14 (87%) peptide 13 (78%) peptide 12 (65%).

Table 5.3: Total number of samples, Mean age of individuals, when and where the samples that used for this part of the work had been collected.

Study panel Description	No. of subjects	Mean age of subjects	Date of collection	Location Or Origin	Clinical features
Qatar study 1st season paired sera (Pre & post) Q1-Q30	30	1year	Nov. 1999 – April 2000	Samples from hospitalised patients at HMC	*ART
Qatar study 2nd season paired sera (Pre & post) Q31-Q35	5	1year	Dec. 2000– April 2001	Samples from hospitalised patients at HMC	ART
Qatar neonatal sera	20	15 days	Randomly	Hospitalised patients from neonatal care unit	No resp. reasons
Adult sera from UK	6	43 years	2002	UK	Lab. Staff

*ART: Acute respiratory tract infection.

5.2.4 Screening peptide reactivity with Qatar study sera

A panel of 35 paired sera from Qatar children before and after natural infection with RSV A or B and 3 neonatal sera from children admitted for non-respiratory problems but which were found to have high anti-RSV neutralizing antibodies (RSV $^-$Ab$^+$) were tested for their reactivity with all 17 peptides (paired sera, and subtype virus were summarised in **Appendix 3**) using solid-phase ELISA that had been previously optimised with human serum (section 5.2).

In attempt to summarize the peptide reactivity, a statistical analysis was undertaken on results of the peptide reactivity of sera taken before infection (OD pre) and after infection (OD post) by showing the percentage of samples where the cut off value was (OD > 0.2) (using SPSS programme).

Figure 5.5 shows the total number of sera from before and after infections that react or do not react with each peptide, and clearly shows that peptides 11, 12, 14, 16 and 17 were recognised by all serum samples taken before and after infection.

Reactivity in the each peptide was generally similar with slightly increased reactivity with post-infection sera (**Figure 5.6**).

Figure 5.5: Peptide reactivity, exposed as % positive cases, where OD % reading was > 0.2.
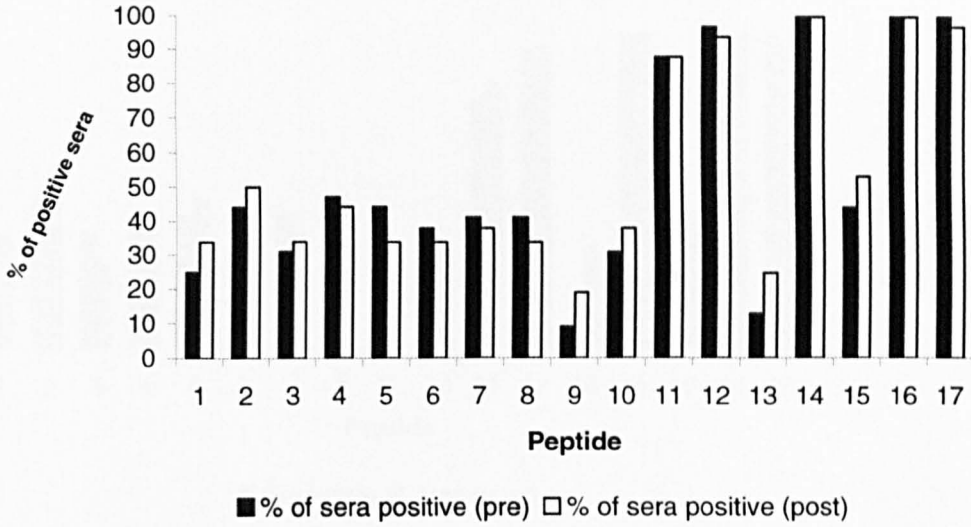
Black columns indicate the percentage of reactivity in samples collected from children before infection, and white columns represent the percentage reactivity in samples collected after infection.

Figure 5.6 (a & b): The mean values of the peptide reactivity (OD) with sera before and after infection with (a) RSV A and (b) RSV B.

(a)
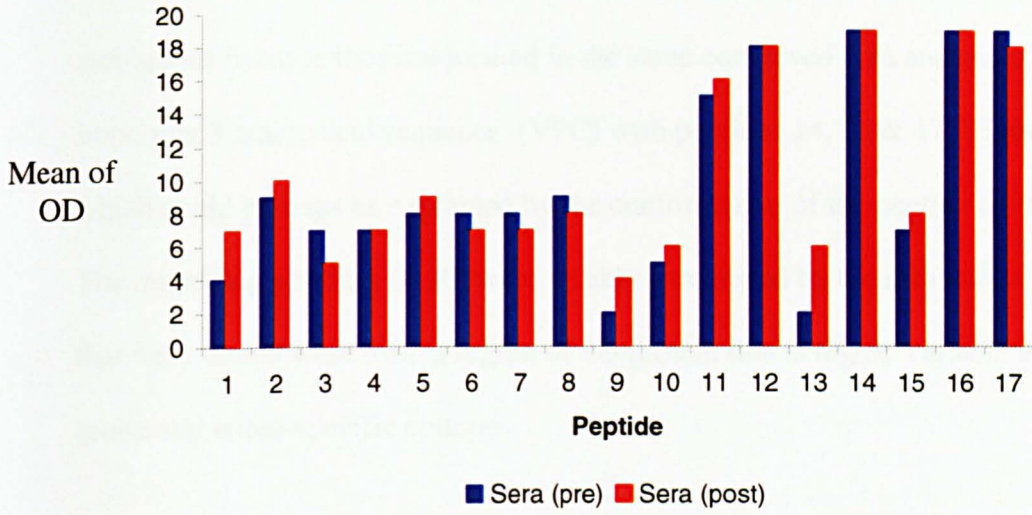
(b)
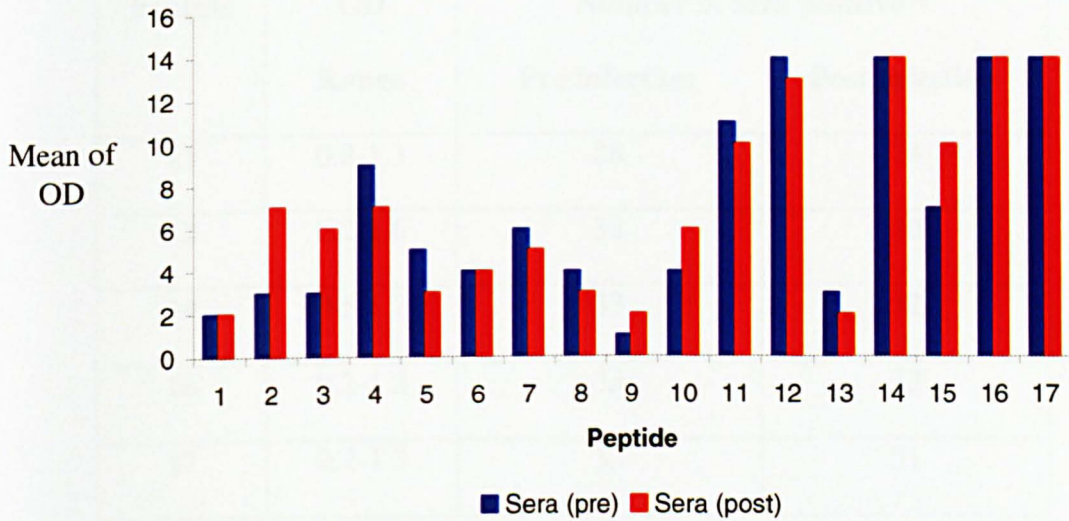
Peptides 14, 16 and 17 have the highest mean OD value reactivity. Peptide 2 reacts strongly with post-infection sera especially with children infected with RSV B.

Majority of sera reacted strongly (ELISA OD in excess of 0.5 at a 1:10 dilution) with 5 of the 17 peptides that are mainly from the conserved area of the G protein (peptides 11, 12, 14, 16 & 17) see **Table 5.4**. The results with peptides 13 and 15 were ambiguous because they are located in the same conserved area and share an important 3 amino acid sequence (VPC) with peptides 14, 16 & 17 (**Figure 5.1**), which could perhaps be explained by the conformation of the peptide on the plate. The remaining peptides (1-10) were weakly recognised by the sera and most of those that were bound were from a region of the protein that is highly variable and contains group and strain-specific epitopes.

Table 5.4: Peptides recognized by sera at dilution 1/10.

Peptide	OD Range	Number of sera positive *	
		Pre infection	Post infection
11	0.2-1.3	28	28
12	0.2-1.6	32	30
14	0.5-1.7	33	32
16	0.2-1.2	33	32
17	0.2-1.3	33	31

* Out of 35 paired sera tested

Peptide 14 shows the highest OD values. There was no difference between reactivity of sera from before and after infection, with peptides (14, 16, 17) from the conserved region of RSV G protein.

5.2.5 Screening peptide reactivity with neonatal and adult sera

Neonatal sera: Initially, only 3 neonatal sera were used, and as shown in **Table 5.5** the neonatal sera did not react with peptides 2,3,5,7 and 9. Antibodies in neonatal sera (mainly maternal antibodies) are important in that they can adversely effect the response to vaccination (as in the case of measles). Thus those peptides that are not recognised by neonatal sera could potentially be used to immunise and thus avoid this interference effect of maternal antibodies. Peptide 2 would seem to be a particularly useful peptide for this purpose since it was recognized by a large number of sera from older children with slightly high OD values (> 0.2) (**Table 5.5**). To confirm this result, further neonatal sera (7) were tested for their reactivity with peptide 2 and also with peptide 14 (which was recognised by most sera from the Qatar study and the adult sera, with high OD values). **Figure 5.7**shows that peptide 2 was not recognised by any of these additional neonatal sera.

Tables 5.5 (a&b): Summary of peptide recognition by Qatar children sera and adult sera.

Peptide	Number of positive Qatar study sera
2	30/70 (43%)
3	21/70 (30%)
5	25/70 (36%)
7	26/70 (37%)
9	9/70 (13%)

a: Peptides recognised by large number of sera from older Qatar study children

Peptide	Adult Sera
12	7/10
13	8/10
14	9/10
15	7/10
16	8/10
17	7/10

b: peptides recognized by most adult sera

Figure 5.7: Screening peptide 2 & 14 reactivity with neonatal sera.
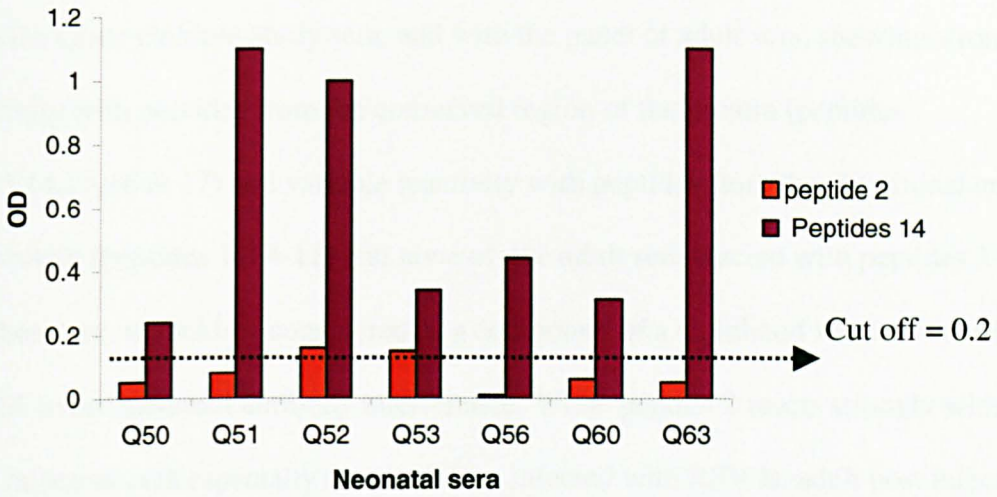
All neonatal sera were unreactive with peptide 2, but 4/7 reacted significantly with peptide 14.

Adult sera: 10 adult sera (4 of which were paired sera taken before and after recent infection with RSV strain B), were tested for their reactivity with all 17 peptides, and representative data are given in **Figure 5.8 A** similar profile of reactivity was seen with the Qatar children study sera, and with the panel of adult sera, showing strong reactivity with peptides from the conserved region of the protein (peptides 12,13,14,15,16 & 17) and variable reactivity with peptides from the C-terminal end of the protein (peptides 1,3,4-11) and none of the adult sera reacted with peptides 2 and 11, therefore, it could be considered as a compound of a childhood vaccine since it would avoid maternal antibody interference. While peptide 2 reacts strongly with post infection sera especially from children infected with RSV B, adult post infection sera reacts better with peptide 16. Of course, these peptides would need to be assessed for their ability to induce virus-neutralising and protective antibody responses in model system before they could be considered as potential vaccine.

Figure 5.8: Screening peptide reactivity with adult sera.
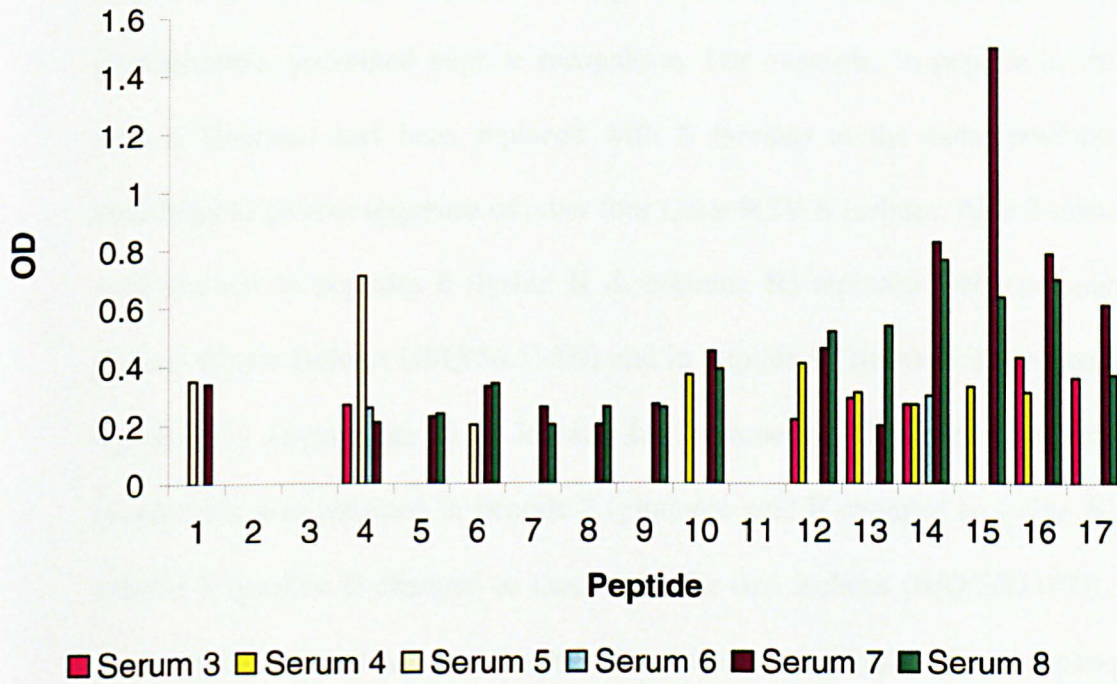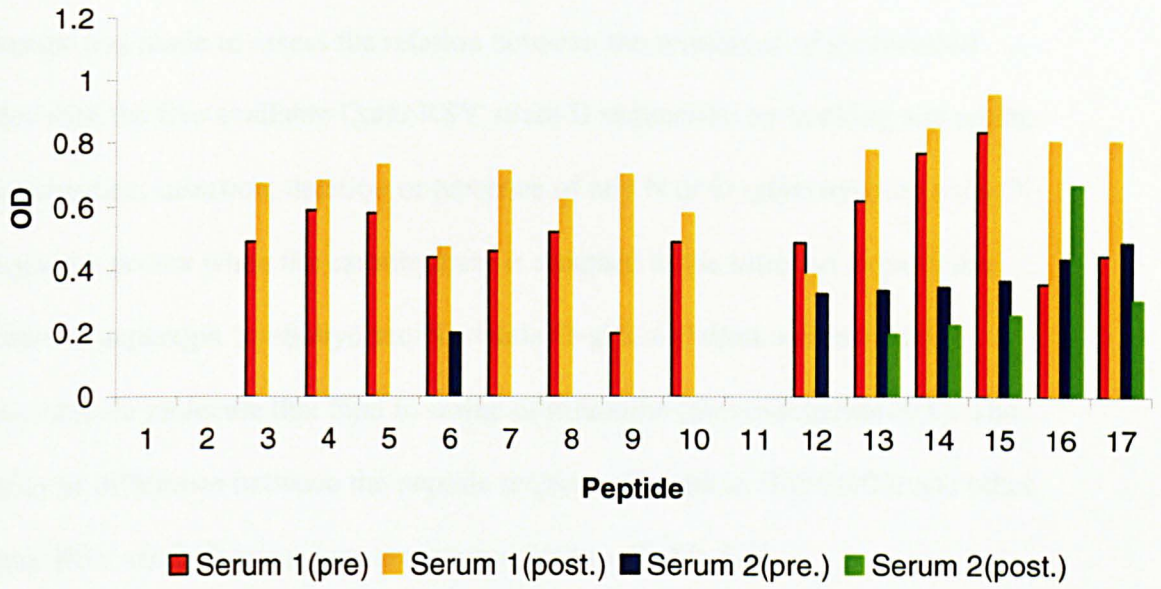
Most of the adult sera reacted highly with peptides 12-17 but did not react with peptide 2 and 11 and reacted weakly with peptides 1,3 and 10.

5.2.6 Relationship between the peptide sequences based on B/Q/28/00 and other sequences of the Qatar strain B viruses.

An attempt was made to assess the relation between the sequences of synthesized peptides with the five available Qatar RSV strain B sequences, by tracking any amino acid substitution, insertion, deletion or presence of any N or O -glycosylation sites (N-glycosylation occurs when the carbohydrate is attached to the nitrogen atom in the side chain of asparagin (carbohydrate-N), while O-glycosylation occurs at any monosaccharide molecule that bind to serine or threonine (monosacharide-S/T). The similarity or difference between the peptide sequence (based on B/Q/28/00) and other the Qatar RSV strain B sequences are summarized in (**Table 5.6**).

From **Table 5.6 (a & b)** Most peptides react with sera from a patient infected with B/Q/33/00) and any sequence change, either as amino acid substitution or glycolysation, prevented peptide recognition. For example, in peptide 2, the amino acid **L** (leucine) had been replaced with **S** (serine) at the same position in the remaining G protein sequence of other four Qatar RSV B isolates. Also 2 substitutions were present in peptides 6 (lysine **K** & arginine **R**) replaced with (threonine **T** & glycine **G**) for isolates (B/Q/5&11/00) and in peptide 9 (lysine **K** & proline **P**) were replaced by (asparagine **N** & leucine **L**) in same two isolates. One amino acid substitution was obtained in peptide 7 (glutamic acid **E** changed to lysine **K**) and in peptide 8 (proline **P** changed to leucine **L**) for two isolates (B/Q/5&11/00). Finally there were 3 amino acid replacements in peptide 10 (lysine **K** to asparagine **N**, threonine **T** to proline **P**, isoleucine **I** to threonine **T** and lysine **K** to asparagine **N**) for the two same isolates (B/Q/5&11/00).

N- Glycosylation sites were present on peptides 1,2 & 3 and O-Glycosylation sites were present on peptides 2,3,4 & 5.

In summary, amino acid changes had occurred in peptides 1 to 10, which may account for the low reactivity of these peptides with the sera from the Qatar study. However, the low reactivity of peptides 11,12 & 13 with sera could not be explained in this way because these sequences were identical for all 5 Qatar RSV B isolates. Only two sera (Q28 & 33) reacted with them. It is possible that these peptides failed to react with antibodies in sera despite the similarity of their sequence, because antibody reactivity was influenced by changes in previous amino acid segments or on their three dimensional structure.

Table 5.6 (a & b): Comparison of peptide sequences of Qatar RSV B strains

Peptides

(a) Serum: serum from children infected with isolate.

* Serum	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
	O	O &N	O &N	O	O	O	O	O &N	O	O							
B/Q/5/00		L→S				K→T R→T	E→K	P→L	K→N P→L	K→N T→P I→T				■	■	■	■
B/Q/11/00		L→S	■			K→T R→T	E→K	P→L	K→N P→L	K→N T→P I→T			■	■	■	■	■
B/Q/18/00		L→S												■		■	■
B/Q/28/00	■	■	■	■	■	■	■	■		■	■	■	■				
B/Q/33/00	■	L→S	■	■	■	■	■	■	■	■	■	■	■				

Yellow squares: serum reacts with peptide (O.D > 0.2 at a dilution of 1:10)

O: O-glycolysation site (monosaccharide –S (Serine)/T (threonine)

N: N-glycolysation site (N (asparagine)-X (amino acid))

□: Amino acid substitution or replacement (Leucine **L**, lysine **K**, arginine **R**, threonine **T**, glycine **G**, proline **P**, asparagine **N**, glutamic acid **E** and isoleucine **I**)

(**a**) The amino acid sequence of peptides shown in bold is the same in all strains. B/Q/33/00 strain has a similar sequence to B/Q/28/00, the source of the synthetic peptide. They differ only in the region of peptide 2 where instead of leucine in B/28 strain, the B/33strain has serine. It seems that the difference is not important because peptide 2 still reacts with serum from a patient infected with strain B/33.

(b)

Peptide	Sequences
2	B/Q/28 – QTPTASEPST**L**NSTQ
	B/Q/5 – QTPTASEPST**S**NSTQ
	B/Q/11 – QTPTASEPST**S**NSTQ
	B/Q/18 – QTPTASEPST**S**NSTQ
	B/Q/33 – QTPTASEPST**S**NSTQ
6	B/Q/28 – T**KE**R**D**TSTSQSTVLD
	B/Q/5 – T**TE**G**D**TSTSQSTVLD
	B/Q/11 – T**TE**G**D**TSTSQSTVLD
7	B/Q/28 – TNPTE**E**PTPKTKERD
	B/Q/5 – TNPTE**K**PTPKTKERD
	B/Q/11 – TNPTE**K**PTPKTKERD
8	B/Q/28 – **P**AKTLKKETTNPTE
	B/Q/5 – **L**AKTLKKETTNPTE
	B/Q/11 – **L**AKTLKKETTNPTE
9	B/Q/28 – KTT**K**KRDPKT**P**AKTL
	B/Q/5 – KTT**N**KRDPKT**L**AKTL
	B/Q/11 – KTT**N**KRDPKT**L**AKTL
10	B/Q/28 – TIKPT**KK**P**TI**KTT**KK**
	B/Q/5 – TIKPT**N**KP**PT**KTT**NK**
	B/Q/11 – TIKPT**N**KP**PT**KTT**NK**

(b): Relationship of peptide sequences between the Qatar RSV strain B isolates. Amino acid substitutions are underlined in B/Q/28/00 and the replacement in the other strains are shown in red.

5.2.7 Assessment of affinity using the inhibition assay

Previous results showed that peptides 14,16 &17 were recognised by a large number of Qatar children and adult sera. These peptides have similar amino acid sequences in the conserved region of RSV B G protein. In an attempt to analyse this peptide antigenicity and to determine if they might be expected to induce a good immune response if tested in future in animal experiments, it was decided to determine the affinity (the tightness of antibody binding to the antigenic determinant) of the antibodies in some sera that react with these peptides. Peptide 14 was chosen initially because it was recognized by a large number of sera with the highest OD values and because it is located in the conserved region of the G protein.

The affinity of an antibody response is a factor of critical biological significance (Steward, 1983) because it represents the strength of binding of antigen and antibody complex and the potentially important epitopes will be those epitopes that can induce and be recognized by antibodies with high affinity.

A number of methods are available for the determination of affinity. In each procedure a system is set up in which antigen and antibody are allowed to come to equilibrium:

$$A b + Ag \Leftrightarrow Ab\text{-}Ag.$$
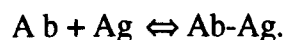
The quantities of free antigen and complexed antigen are then measured without disturbing the equilibrium. There are several ways of doing this. Some separate the free antigen from the bound antigen by physical methods such as dialysis, gel filtration, centrifugation and selective precipitation. Others use change in the fluorescent properties of the complexed antigen and antibody.

Data from these systems is analysed by applying the Law of Mass Action to give the equilibrium (affinity) constant, K:

$$K = \frac{[Ab\text{-}Ag]}{[Ab]\,[Ag]}$$

Where [Ab-Ag] is the concentration of complexed antigen, [Ag] is the concentration

of free antigen and [Ab] is the concentration of free antigen binding sites at

equilibrium. When half the binding sites are occupied by antigen, [Ab] = [Ab-Ag], so

it follows that K = 1/[Ag]. In other words, a high-affinity antibody (one with a high

K) only requires a low antigen concentration to achieve binding of antigen to half its

combining sites, whereas a low – affinity antibody requires a much higher

concentration of antigen to achieve this. Inhibition EIAs have been used in this work

to obtain estimate of antibody affinity in some sera from Qatar. Serum samples were

titrated to determine the required inhibitor dilution and the amount of peptide to be

used in the affinity assay was determined

A measure of the affinities of human anti – RSV antibodies to the relevant peptide

was determined by a solid – phase enzyme inhibition assay (Rath et al., 1988).

Appropriate dilutions of serum in blocking buffer were preincubated with 0.5 log $_{10}$

dilutions of inhibiting peptide at 37 °C for 1 hour. The relative antipeptide antibody

affinity was determined as the reciprocal of the molar concentration that gave 50%

inhibition of the binding observed in the absence of inhibiting peptide.

Determination of affinity maturation of antibodies reactive with peptide 14:

In order to determine if there is affinity maturation of the antibodies to peptide 14 (i.e.

a progressive immune in affinity with them), the inhibition assay was used with

peptide 14 as the competitor peptide, RSV Ag as positive control and peptide 9

(weakly reactive peptide) as negative control. A panel of sera obtained before

infection and sera from the same individuals taken after infection was used for this experiment **Table 5.7** and **Figure 5.9**.

Table 5.7 was constructed from results of the serum neutralizing assay (see following chapter). The average maximum OD for each serum (without inhibitor) was calculated. The log nMolar concentration of free peptide was calculated, using information from **Table 5.3.**

Because the molecular weight of RSV antigen is unknown, the antigen concentration in $\mu g/ml$ was calculated. N.B. As during the assay a 2 fold dilution of the peptide and antigen was made by the addition of serum, the final concentration was half that of the calculated concentration.

Graphs 5.9 were plotted of the OD against the log of nMolar concentration of peptides. From these graphs the log nMolar concentration of peptide that caused 50% inhibition was calculated. The anti-log of this figure was taken and the nMolar concentration converted to Molar concentration. To calculate the affinity of the antibodies, the reciprocal of this Molar concentration was taken and the result expressed as liters per mole.

Peptide 14 (from RSV B G protein) could represent an important epitope stimulating high affinity anti-RSV antibodies **(Table 5.7)**. Affinity values of 2 sera after infection (Q28 (B) and Q37 (A) were higher than before infection (i.e. affinity maturation). However, serum Q20 (B) has a high neutralization titre and its affinity value against peptide 14 after infection is lower than before infection (i.e. no affinity maturation). Larger amount of RSV antigen were utilized by Q20 (B) serum collected after infection than serum collected before infection   to prevent binding to the antigen coated plate. It is possible that these neutralizing antibodies were mainly directed to other proteins (e.g. F protein) or to a conformational epitope. Another possibility is

that neutralizing antibodies were mainly produced by the primary response and other

neutralization antibodies were produced by the secondary response (more by memory

cells).

These results (**Figure 5.9 & Table 5.7**) suggest that peptide 14 probably represents a

dominant epitope because it inhibits RSV-anti RSV binding nearly as well as does the

RSV antigen.

The affinity of the RSV antigen by the serum was not calculated. Since peptide 9 did

not inhibit the binding of the serum to RSV antigen it is unlikely that peptide 9

represents a dominant epitope.

Figure 5.9: Inhibition assay by using RSV antigen, peptide 14 and peptide 9 as inhibitors.

(a)
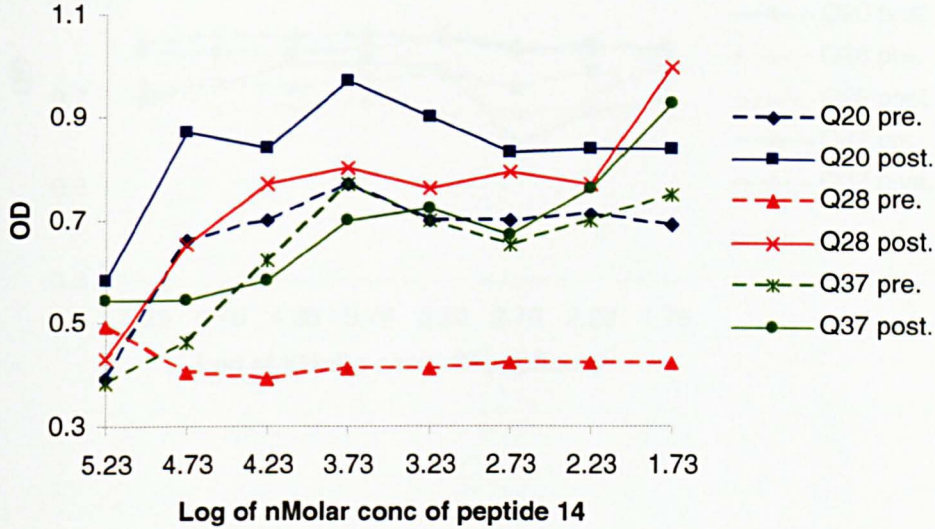
(b)
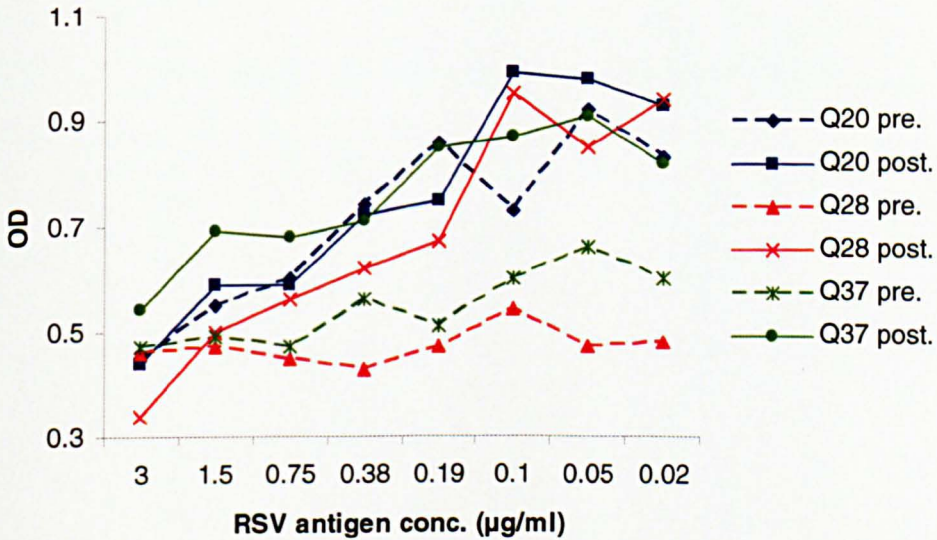
(c)
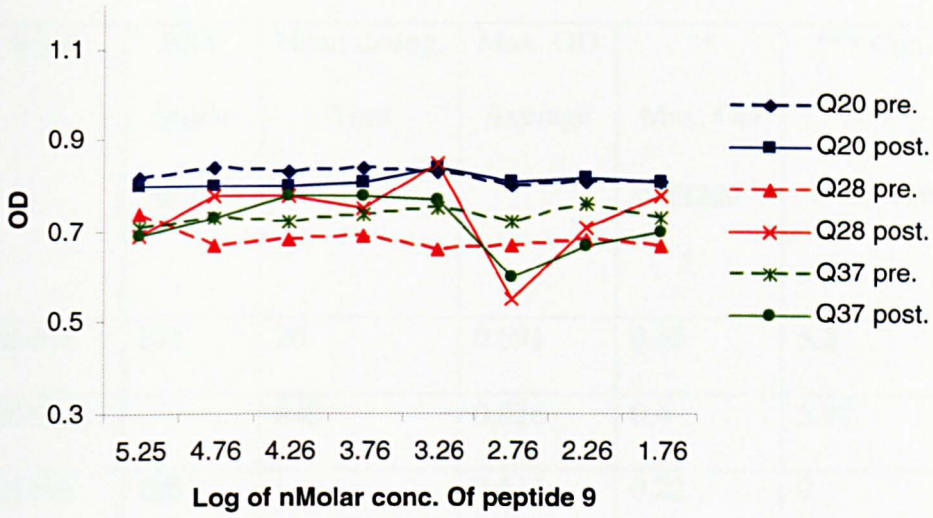
(a) Inhibition of binding of RSV antigen by human sera with free peptide 14. **(b)** Inhibition of binding of RSV antigen by human sera with RSV antigen. **(c)** Inhibition of binding of RSV antigen by human sera with free peptide 9.

These results suggest that peptide 14 probably represents a dominant eiptope because it inhibits RSV-anti RSV binding nearly as well as does the RSV antigen.

Table 5.7: 50% inhibition assays by using RSV antigen, peptide 14 peptide and 9 as inhibitors.

(a)

Sera	RSV Strain Age/*M	Neutralizing. Titre	Max. OD Average	** Max. OD Average 2	*** Con. 50% inhibition	**** Peptide Affinity
Q20 Pre	B/4	20	0.691	0.35	5.2	0.63×10^4
Q20 Post.		640	0.826	0.4	5.92	0.12×10^4
Q28 Pre	B/6	5	0.444	0.22	9	0.1×10^4
Q28 Post		1280	0.791	0.39	5.4	0.39×10^4
Q37 Pre	A/10	80	0.636	0.32	4.86	1.38×10^4
Q37 Post		320	0.861	0.43	4.44	3.66×10^4

*   Months
**   This value was used for the inhibition assay
***   Log of nMolar of free peptide 14 concentration that give 50% inhibition
**** The reciprocal M^{-1}

(a) Inhibition of binding of RSV antigen by human sera with free peptide 14.

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Table 5.7: 50% inhibition assays by using RSV antigen, peptide 14 peptide and 9 as inhibitors.

(b)

Sera	RSV Strain Age/M	Neutralizing. Titre	Max. OD Average	Max. OD <u>Average</u> 2	RSV antigen conc. (μg/ml) for 50% inhibition
Q20 Pre	B/4	20	0.845	0.42	1.32
Q20 Post.		640	0.886	0.44	1.48
Q28 Pre	B/6	5	0.459	0.23	1.1
Q28 Post		1280	0.959	0.48	2.06
Q37 Pre	A/10	80	0.592	0.29	1.46
Q37 Post		320	0.841	0.42	1.6

(b) Inhibition of binding of RSV antigen by human sera with RSV antigen. There is no Inhibition of binding of RSV antigen by human sera with free peptide 9.

5.3 Discussion:

Screening of serum from children (the important target population) for reactivity to 17 RSV B – G protein peptides showed that there were antibodies to the conserved and C – terminal region of this protein in the samples collected from children before and after infection, even when the target antigen used in the assay was a linear peptide that was not glycosylated. The N-terminal section of the G protein was not studied because this part contains all the structural information required for efficient membrane insertion and cell surface expression. The N-terminal 71 amino acids of the G protein of RSV A strain failed to induce significant levels of G-specific antibodies in an other study (Olmsted et al., 1989) and as shown in **Figure 4.11** this part of the protein has a low antigenic index (as assessed by protein programme of DNASTAR database). The strictly conserved region of the protein was covered with 2 extra peptides (16 & 17).

The results obtained from screening the serum of Qatari children collected before and after infection showed a similar profile of peptide recognition. Also, sera from children infected with strain A, can bind peptides from the G protein of strain B (cross-reactivity), for examples peptides 14, 16, and 17.

The majority of serum samples from all children infected were reactive with peptides 14, 16, 17, 12 and 11. This suggests that these peptides could represent epitopes in the central region of the G protein that could be important targets for the induction of protective antibodies. From the amino acid sequences of the 5 peptides, it can be seen that they share some sequences with the strictly conserved 13 amino acid sequence present in all RSV strains (164-176). In particular, the important 3 amino acid sequence (VPC) is present in peptides 14, 16 and 17, this sequence has been found to

be important in several studies (Simard et al., 1997) and (Akerlind-Stopner et al., 1990).

Peptide 2 could be a potential candidate for use in a peptide – based vaccine for children under 6 months of age to avoid interference with maternal antibodies (Munoz & Gelzen, 2003; Weisman, 2002) because it was not recognized by any neonatal or adult study sera and it was recognized by 43% of serum samples from children over 1 month of age.

However, because this peptide derives from the C-terminal region of the G protein of RSV which is highly variable (Cane et al., 1996) and this sequence in RSV B/Q/ 28/00 contains one amino acid substitution and N & O-glycosylation sites, it may not be a good vaccine choice.

The low peptide binding reactivity of peptides 1 to 10 with human sera could be a result of the heterogeneity of these peptide sequences, caused by either amino acid substitution or glycosylation. Similarity between the sequences of peptide14, 16 & 17 with the sequences of the infecting type for all Qatar isolates are rise to almost the same antibody recognition profiles. This is consistent with the finding that the pattern of peptide reactivity is closely related to the infecting genotype in animal models (Cane, 1996; Sullender, 1999).

Peptide 14 was strongly recognized, highlighting the potential importance of residues 150 – 165 of the G protein from RSV B strain. The fact that peptides 14, 16 & 17 are strongly bound by sera from children may indicate that epitopes in this region of the protein are importance in protection against RSV.

A relatively large proportion of antibody responses to viral proteins are possibly directed towards conformational rather than to linear B – cell epitopes (Cane et al., 1996). This fact can explain the low antibody recognition of peptides 13 & 15, which

share some, sequences from the conserved region of protein G that has high antibody binding reactivity and lack of antibody recognition of peptide 11 by adult serum. The responses of babies to the variable of region of the G protein appeared to be related to some extent to the age of the child as has been reported in relation to affinity maturation of the response (Murphy et al., 1986; Rath et al., 1988; Atabani et al., 1997). In order to determine the antigenicity of one representative peptide from the conserved region of protein G of RSV B strain by inhibition assay and to determine if there is any affinity maturation of antibody binding peptide 14 was chosen because this peptide was recognized by the majority (93 %) of sera.

There was demonstrable affinity maturation in post-infection samples Q28 and Q37 but not Q20. However, there was evidence of a secondary response in Q20 since the binding levels were much higher in the post-infection sample. These data suggest that peptide 14 could contain important epitopes that are recognized by high affinity antibodies post infection which inhibit antibody binding to RSV antigen.

However, these results with peptide 14 are only preliminary and further immunization studies will be necessary and other peptides should be also studied (e.g. peptide 16, 17 and 2) to see whether they also are recognized by antibody with high affinity.

It should be emphasized that even if peptides are bound with high affinity by all anti-RSV sera it does not necessarily follow that they will be good vaccine candidates. This can only be indicated after extensive studies of their immunogenicity and antigenicity and their ability to induce virus-neutralizing and protective response in animal models.

6 Chapter 6: Analysis of the neutralization of RSV by sera from Qatari children

6.1 Introduction:

Immune responses to RSV have been extensively investigated and the induction of neutralizing antibodies has been associated with development of host protection. In infants, the presence of higher levels of maternal antibody are associated with increased levels of protection (Anderson & Heilman, 1995; Melero et al., 1997), however, infection is still possible even in the presence of maternal antibodies. Serological data and evidence from nucleotide sequencing indicate that major antigenic and amino acid sequence differences are found in the G glycoproteins of group A and B viruses, while the F glycoproteins of both group are more highly conserved. Recurrent infections with RSV occur throughout life. The importance of strain variation to protective immunity is less clear. Only a limited number of amino acid changes are required to produce a phenotype resistant to neutralization antibodies (in vitro) (Cane et al., 1994). Garcia-Beato et al., (1996) have shown that infection of different human epithelial cell lines with RSV, following passage in cell culture, revealed changes in the reactivity of the G glycoprotein with anti-G monoclonal antibodies. These results highlight the importance of cell-type specific modifications for the antigenicity of RSV G glycoprotein and raise questions about the actual antigenic structure of this molecule when RSV replicates in the respiratory tract.

Some of the epitopes recognized by human antibodies that developed after natural infection have been mapped in the G protein by testing reactivity of convalescent sera either with synthetic peptides (Cane, 1997) or with protein segments of the G molecule expressed in bacteria as a fusion protein (Cane et al., 1996). Most of these experiments were performed with the laboratory-adapted RSV A strains as previously mentioned in Chapter 5.

Few data are available on the immune responses to RSV B strains, including recently isolated RSV B strains, and little or no information is available concerning which G protein epitopes are recognized. Therefore, the main purpose of this part of this thesis was to determine whether there was a correlation between peptide binding activity to RSV B peptides in vitro and the titres of neutralizing anti-RSV A and B strain antibodies.

Several methodologies have been described for the detection of RSV neutralizing antibody that are based on pretreatment of the virus with a dilution of monoclonal or serum antibody followed by infection of cell monolyers. Residual RSV infectivity is then determined by either a plaque reduction assay or assessment of the reduction in RSV antigen expression. In this part of the project, a modification of the micro neutralization method of (Anderson et al., 1985), as described in the Materials and Methods section 2.24 was used.

The objective of this part of work to:

- Develop a neutralizing antibody assay using lab adapted and recent Qatar isolates.

- Determine whether or not neutralizing antibody titre is correlated with peptide reactivity.

RSV neutralization activity in serum samples was evaluated in paired sera taken before and after infection in young children and in neonatal sera (containing predominantly maternal antibodies). Neutralizing antibodies present in each serum to 3 RSV strains, two RSV A strains (one laboratory adapted strain A2 and the other a recent Qatar isolate A/Q/10/00) and one laboratory adapted B strain N2 were tested to determine the influence of strain and genotype diversity on anti-RSV antibody activity.

6.2 Results:

6.2.1 Growth of viruses:

Virus isolates were made from clinical materials from 2 seasons of the Qatar study group. The growth of virus from samples was assessed using Hep 2 cells and noting the appearance of syncytia and cytopathic effects between 3-5 days post inoculation (Figure 6.1 & 6.2). Considerable variation was noted between the clinical isolates in their ability to form large syncytia and to induce giant multinucleate cells. However, this observation of phenotypic variation in strain growth has not been followed up.

Figure 6.1 (a& b): Low-power view of continuous line of hep-2 cells infected with A/Q/10/00
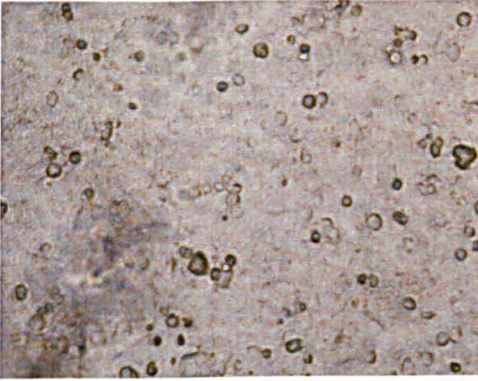
a
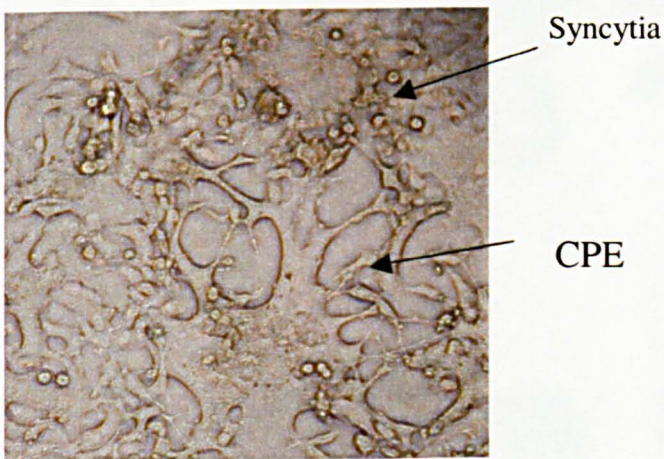
b

Figure 6.1.a Low –Power view of continuous line of hep-2 cells inoculated with 2% MEM media (no virus) as negative control after 72 hours incubation (x 40).
Figure 6.1.b Hep-2 cells infected with RSV positive clinical sample (Qatar/RSVA/2000/10) after 72 hours incubation, multinucleate giant cell (syncytia) and cytopathic effect resulting from infection with RSV (x 40).

Figure 6.2: Electron photograph of (RSV A/Q/10/00) isolated from Qatar after 72 hours incubation and fixed by 3% phosphate buffered gluteraldhyde. (Courtesy of Mr. Wagner, Northern General Hospital).

i. Different maturation stages of RSV infectious cycle in hep2 cells (x 114,100).
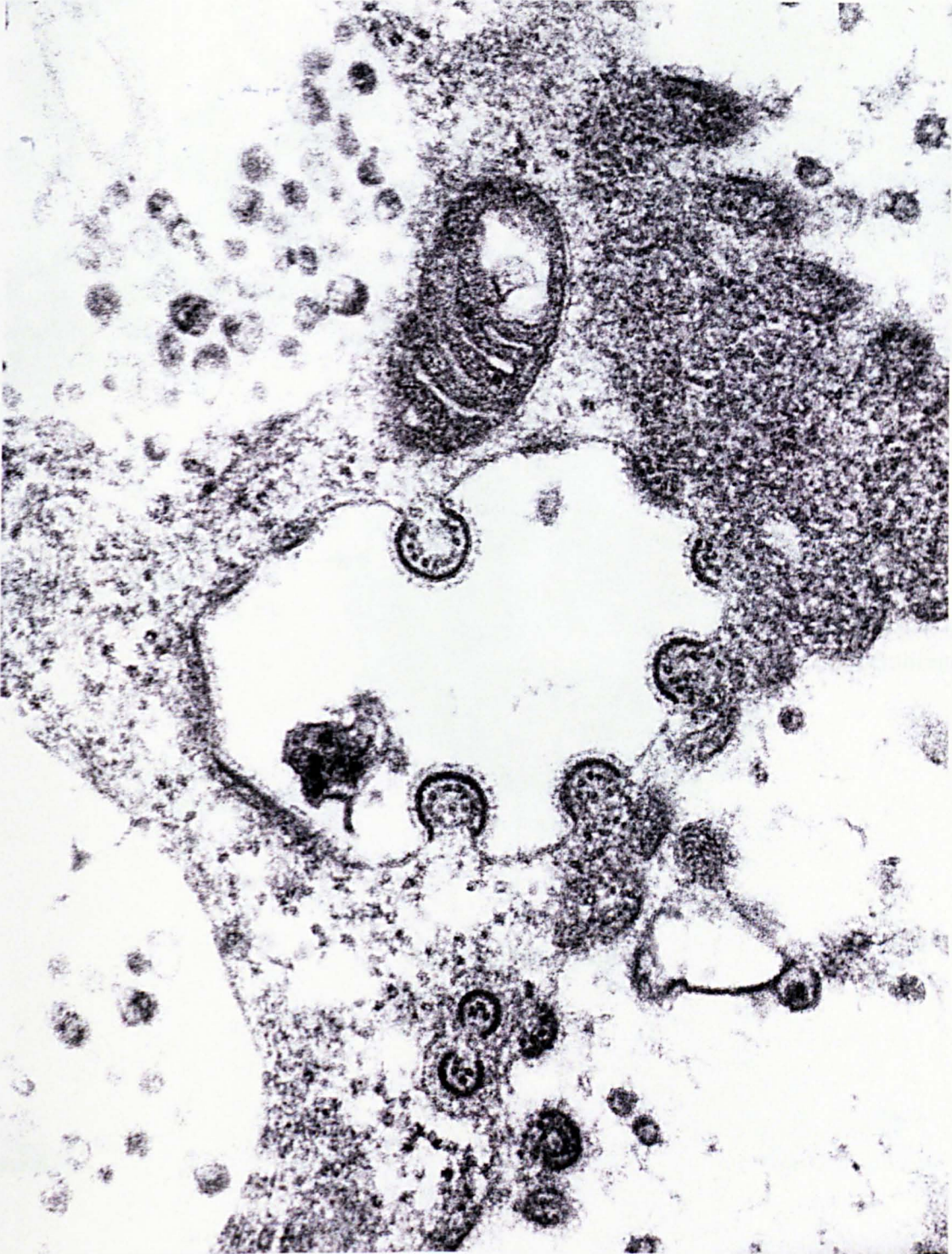
Figure 6.2:

ii. Budding Virion (x 555000).

Envelope spikes (SH, G, & F)

Infected hep-2 cell membrane

Lipid layer (Matrix M, M2)

Viral nucleocapsid (N, P& L)
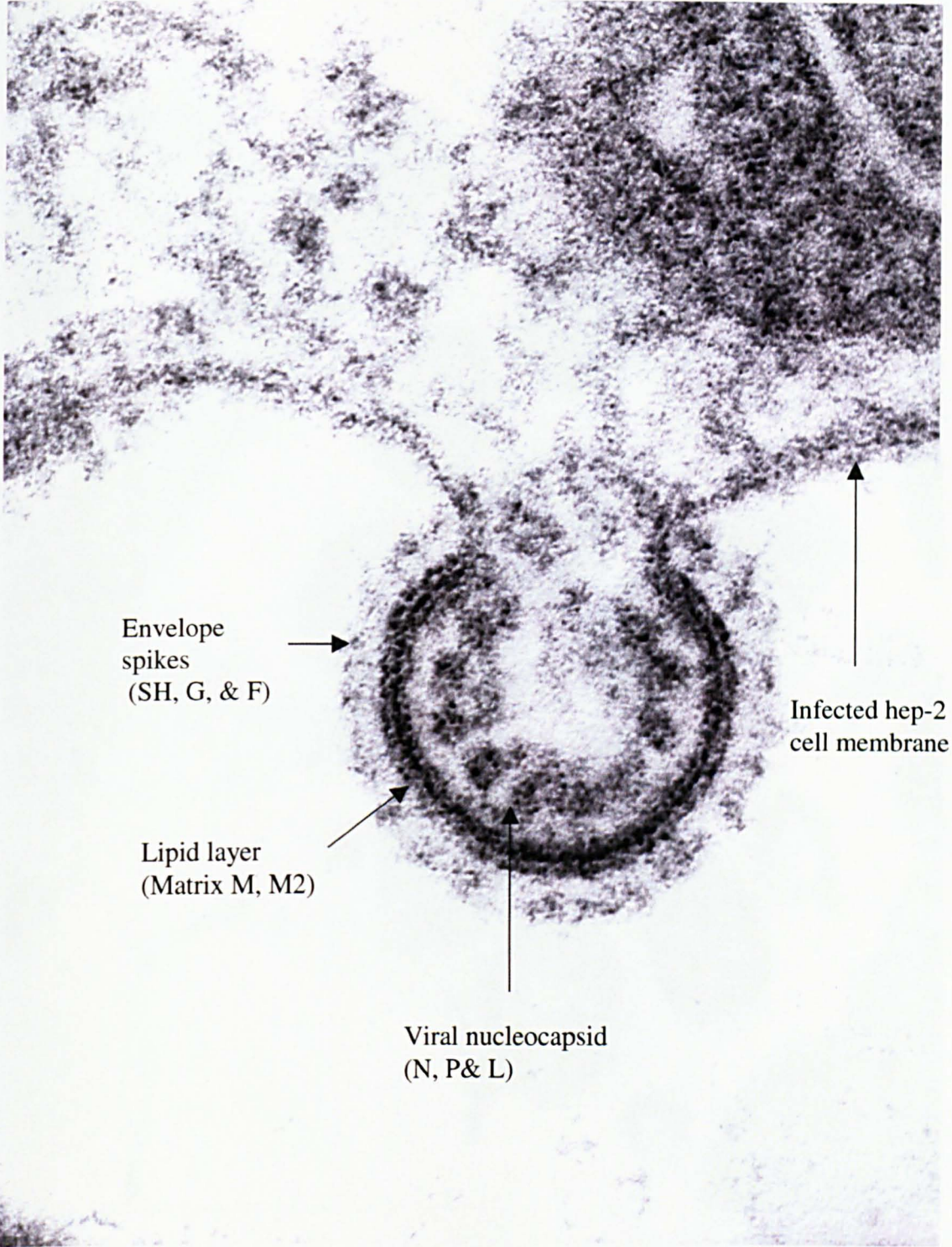
Figure 6.2:
iii. Budding of two type of virion (Longitudinal & Circular particles) (x 248,418).
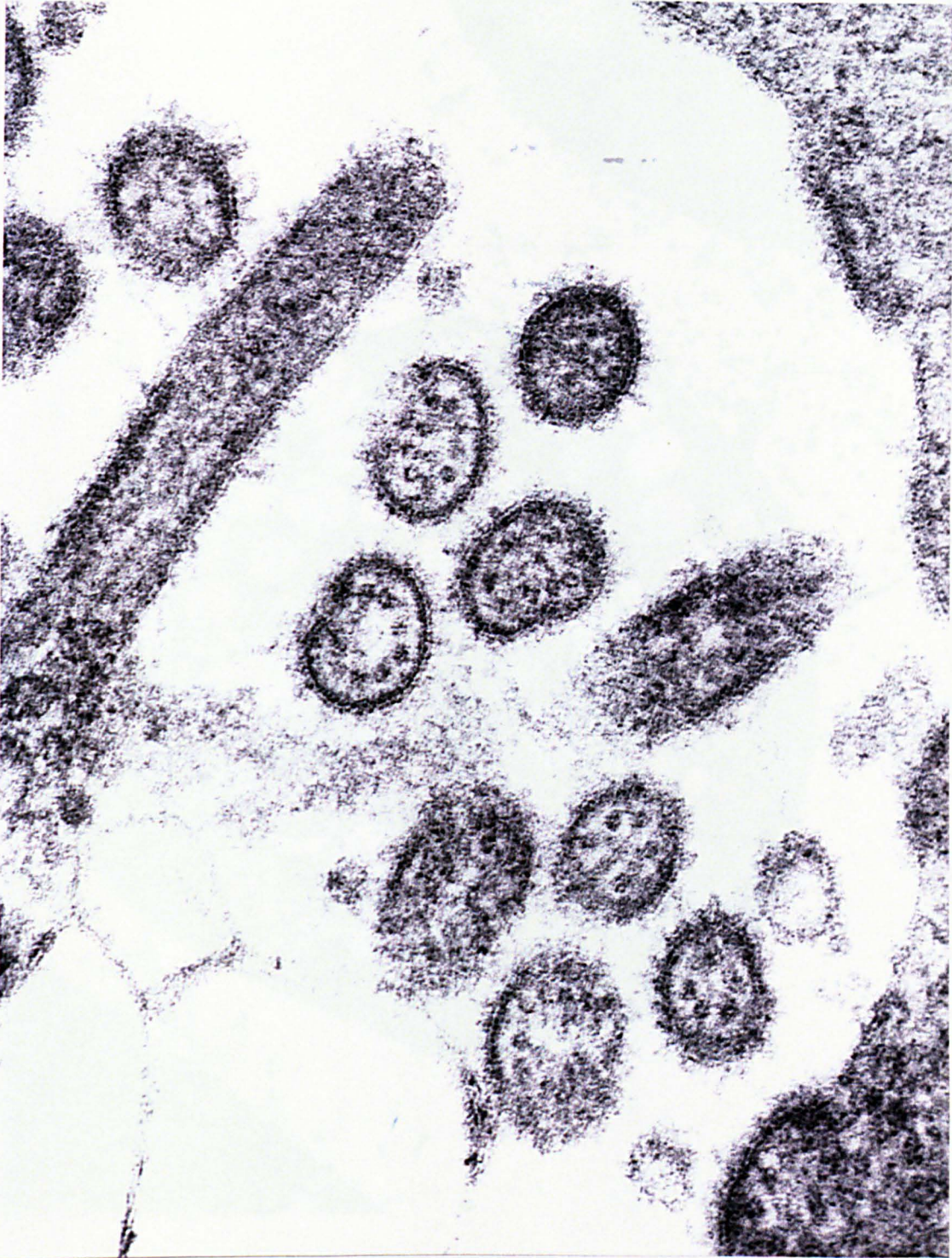
Figure 6.2:
iv. Virus leaving a cell by lysis or budding (x 11,124).

Budding

Lysis

6.2.2 Optimization of neutralization assay:

As described previously in the Materials and Methods, a selection of both commercial and non-commercial antibodies, incubation periods and fixation methods were tested before finalizing a micro-neutralization test for field viruses that could be used with study sera

A panel of 14 primary anti RSV antibodies (monoclonal and polyclonal) was evaluated using the cell-based ELISA test to titrate RSV study controls (RSV A A2 & RSV B N2) to determine which antibody was most suitable for use as a detecting antibody (primary antibody) in virus neutralization tests (**Table 6.1& Figure 6.3**)

It was observed that some anti-RSV antibodies gave brown foci with clear background with RSV A only and at a high working dilution, but not with RSV B, and vies versa.

The optimum incubation period was 3 days for most RSV A & B isolates with the development of clear syncytia and cytopathic effects on the Hep2 cells. For all tested viruses the best fixation method was with methanol hydrogen peroxidase (2%) for 20 minutes (**Table 6.2**).

Unfortunately, of 10 Qatar RSV A isolates and the laboratory –adapted A2 strain tested, only the A/Q/10/00 and the A2 strains were detected by the commercial antibodies. In addition, the commercial antibodies detected none of the 5 Qatar RSV B isolates and only the laboratory-adapted RSVB N2 strain was recognized using the antibodies available for this study. This limited the work, which could be done on neutralization using Qatar B strains.

Table 6.1: Evaluation of panel of primary anti-RSV antibodies by cell based ELISA

1Reactivity	2Immunogen	3 Designation	Animal Source	RSV A2	RSV BN2	RSV AQ/10/00	RSV BQ/28/00
4 F 1b	Long	MAB 8581	Mouse	1/5000	1/1000	No reaction	No reaction
5 G 12	A2	MAB 8582	Mouse	1/9000	No reaction	1/5000	No reaction
G 4	A2	MAB 8583	Mouse	1/1000	No reaction	No reaction	No reaction
G 12	A2	MAB 8584	Mouse	1/9000	No reaction	No reaction	No reaction
G 3b	A2	MAB 8585	Mouse	No reaction	No reaction	No reaction	No reaction
G 5a	Long	MAB 8586	Mouse	1/7000	No reaction	1/1000	No reaction
G	A2	MAB 8593	Mouse	1/9000	No reaction	No reaction	No reaction
F	A2	MAB 8594	Mouse	1/1000	No reaction	No reaction	No reaction
F	A2	MAB 8598	Mouse	No reaction	No reaction	No reaction	No reaction
F	A2	MAB 8599	Mouse	1/1000	1/1000	No reaction	No reaction
F, G & 6 P	A2	MAB 858-4	Mouse	No reaction	1/5000	No reaction	No reaction
47-46 KDA F	A2	MAB 858-1	Mouse	No reaction	1/5000	No reaction	No reaction
89,KD G	A2	MAB 858-2	Mouse	No reaction	1/5000	No reaction	No reaction
Polyclonal	A2	AB 1128	Goat	1/5000	1/5000	1/2000	No reaction

[1]RSV antigen reacts with antibodies, [2] Immunogen: The origin of the virus either RSV A Long strain or A2, [3]All antibodies from Chemicon, [4] Fusion protein, [5]Glycoprotein, [6] nuclear protein.

Figure 6.3: Typical micro neutralization assays.
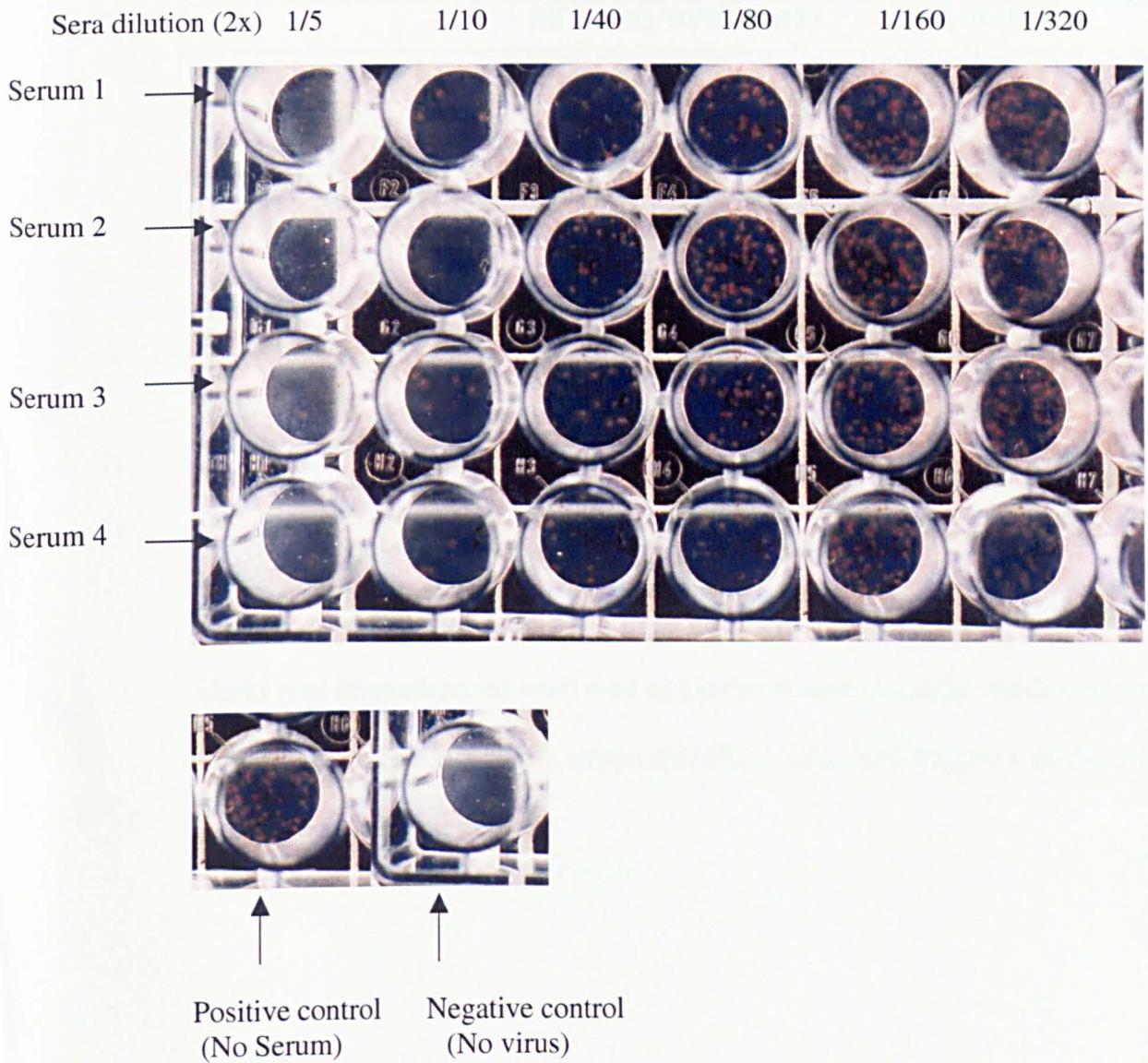
The positive control is virus (A/Q/10/00, 50 pfu/well) without sera. Serum 2 has a neutralizing antibodies titer of 1/40, and serum 4 has titre of 1/80.

Table 6.2: Optimal neutralization virus titration factors

	RSV A (Q/10/00 & A2)	RSV B (N2)
Incubation period	3 days	3 days
Fixation method	Methanol/H_2O_2 (2%)/ 20 minutes	Methanol/H_2O_2 (2%)/ 20 minutes
Primary antibodies	Mab 858-4	Mab 858-2

6.2.3 Final RSV micro neutralization assay:

Antiserum controls produced by inoculating rabbits with the same control strains (2

weeks post immunization) were used as a positive neutralizing antibody control

Figure 6.4. From this figure it can be seen that 2 fold dilutions give a range of results

that can be easily interpreted.

Figure 6.4: Neutralizing of RSV by rabbit anti-RSV serum (section 2.19) and determination of optimal dilution of sample Q1 for use in neutralization assay
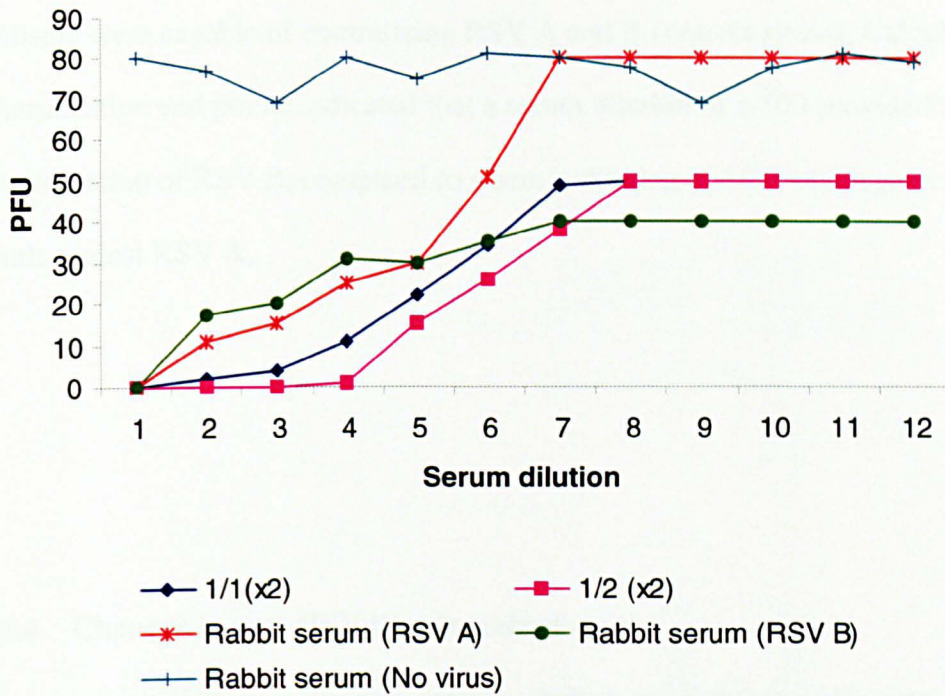
- 1/1 serum collected before infection, 1/2 serum collected after one month of infection from same child infected with A strain.

- (2x) 2-fold dilution.

A neutralization assay was developed for RSV A2 strain and RSV B N2 based on the

cell based EIA methodology, using Mab 858-4 and 858-2 as primary antibodies for

RSV A and B, respectively. The negative controls were culture medium without

antibody or without virus. Results using these antisera are displayed in Figure 6.4

Antisera were capable of neutralizing RSV A and B controls strains, Calculation of

neutralization end points indicated that a serum dilution of 1/100 provided 50%

neutralization of RSV B, compared to a serum dilution of 1/50 which gave a similar

result against RSV A.

6.2.4   Changes in anti-RSV titre in paired sera:

The reactivity of 36-paired serum samples (before and following infection) with 3

RSV viruses (RSV A Q/10/00 (recent isolate) andA2 (lab. Adapted strain) and RSV B

N2) was assessed (**Figure 6.5 a, b, c** and **Table 6.3**), the results of individual sera are

shown in **appendix 9**. The lowest neutralizing antibody titre was observed in acute

sera (range from 1/5 to 1/160). In all acute sera neutralizing antibody titres were

below 200, while most convalescent sera neutralizing antibody titres were above 320.

From this observation it could be suggested that the cut off level for protection was

between a neutralizing antibody titre of 200-300.

Figure 6.5 (a, b, c): Anti-RSV neutralization titre in paired sera from children either infected with strain A or B.

(a)
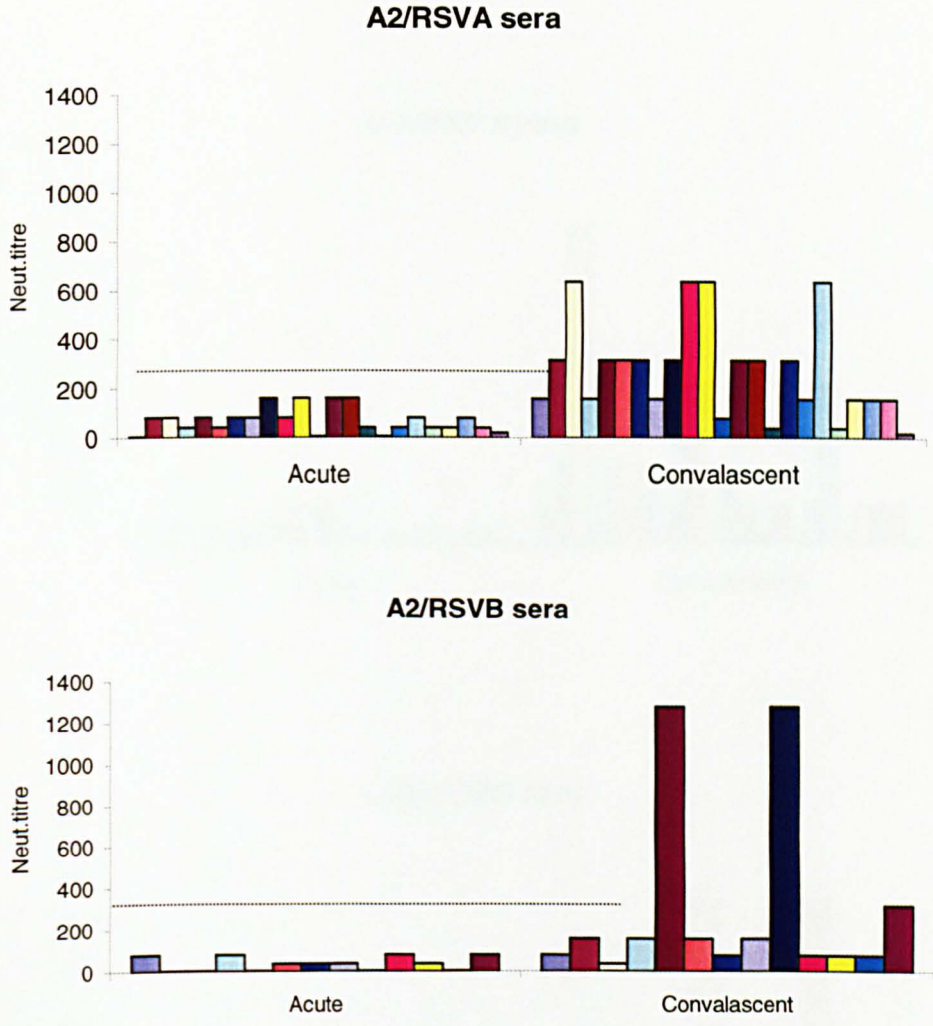
A2/RSVA sera

A2/RSVB sera

Figure 6.5:(a, b, c): Anti-RSV neutralization titre in paired sera from children either infected with strain A or B.

(b)
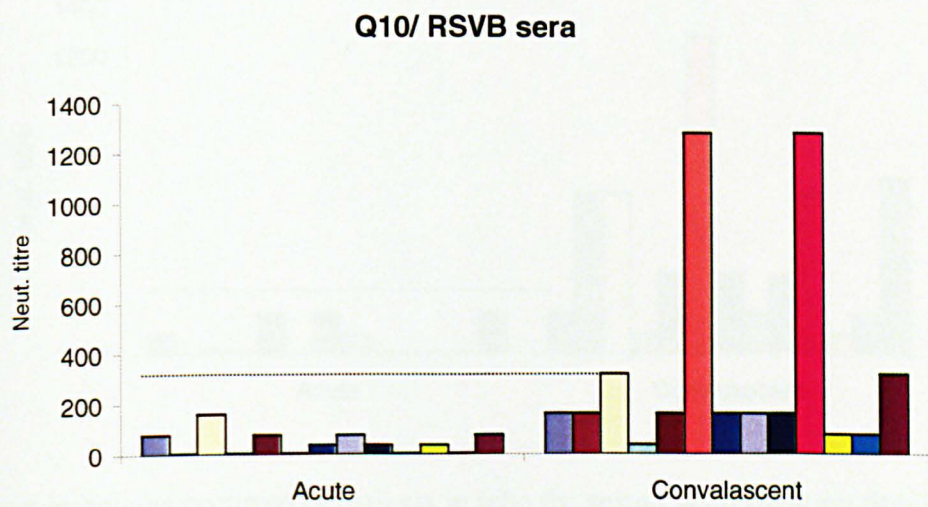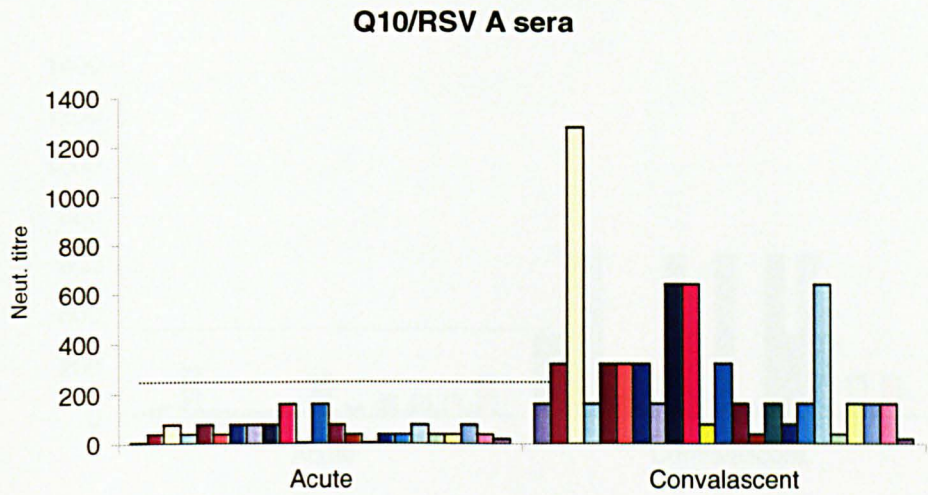
Q10/RSV A sera

Q10/ RSVB sera

Figure 6.5:(a, b, c): Anti-RSV neutralization titre in paired sera from children either infected with strain A or B.

(C)

BN2/RSV Asera

BN2/RSVB sera
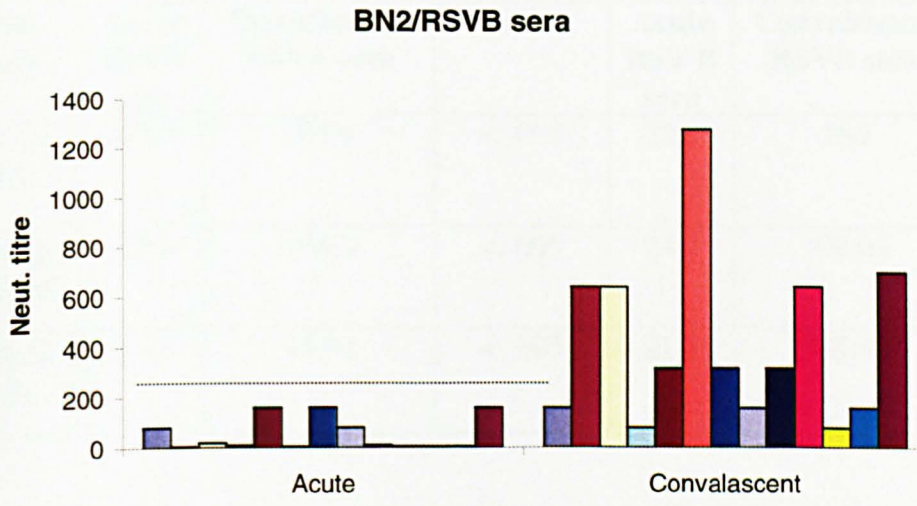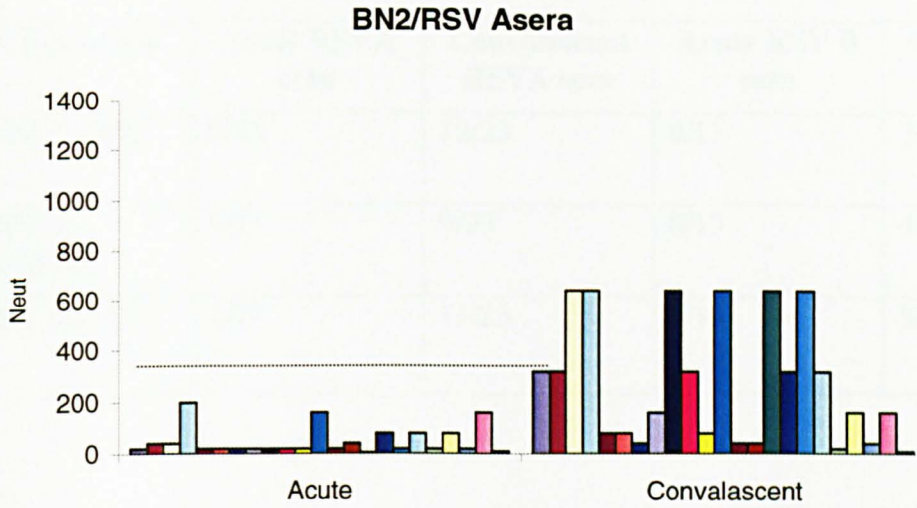
Most infections occurred in patients in who the serum neutralization titre was below 320.

Table 6.3: Number of sera with titre > 200 level of neutralizing titre among acute and convalescent sera of Qatar study children group (a) and their GMT mean and P value (b).

(a)

Virus strain	Acute RSVA sera	Convalescent RSVA sera	Acute RSV B sera	Convalescent RSVB sera
RSV A (A2)	0/23	12/23	0/13	3/13
RSV A (Q10/00)	0/23	9/23	0/13	4/13
RSV B (N2)	1/23	11/23	0/13	8/13

(b)

Virus strain	Acute RSVA sera	Convalescent RSVA sera	P value	Acute RSV B sera	Convalescent RSVB sera	P value
RSV A (A2)	47.9	203.6	<. 005	22.2	160	<. 005
RSV A (Q10/00)	42.5	168.0	<. 005	24.7	198.04	<. 005
RSV B (N2)	31.7	155.2	<. 005	21.1	305.49	<. 005

Convalescent sera with high level of neutralizing antibodies (>200) were more often seen from children infected with RSV B strain than from children infected with RSV A strain especially when the challenge virus was RSV B (N2) strain (**Table 6.3**). Sera with high neutralizing antibody titres show cross reactivity and can neutralize viruses from different groups at high working dilutions. For example, serum samples Q10, 21 & 37 obtained from children infected with RSV A could neutralize RSV B N2 at dilutions of 1/320, 1/640 & 1/320 respectively. In addition, some sera (Q2, 28, 43) from children infected with RSV B could neutralize virus A/Q/10/00 at a high titre 1/1280, 1/1280 & 1/640, respectively. These results suggest that the titre of neutralizing antibody appears to be more important than strain diversity. Also there were no significant differences in neutralizing antibody reactivity when either the recent RSV A isolate (A/Q/10/00) or the laboratory-adapted strain (A2) were used.

6.2.5   The effect of age on RSV neutralizing antibody titre in sera before and after infection:

The relationship between the titre of RSV neutralizing antibody from children (before and after infection) to the child's age at the time of molecular-proven infection was studied in 36 paired sera from the Qatar study children and the neonatal group consisting of 20 sera from children admitted to hospital for non-respiratory symptoms at an age of less than one month. Most of the Qatar study children and neonatal children who were less than 1 month had high titres of neutralizing antibody in their sera (mainly maternal antibodies). This suggests that children are born with a high neutralizing antibody titre (above 320) and after one month the titre decreases and the children become susceptible to infection. **Figure 6.6. a.**

Figure 6.6:(a, b, c, d): Comparison of RSV A & B neutralization antibody titre in individual Qatari children acute serum samples from children challenged with AQ10 strain (a & c) or challenged with BN2 strain (b & d).

(a) AQ10/ RSVA sera

(b) AQ10/ RSVB sera
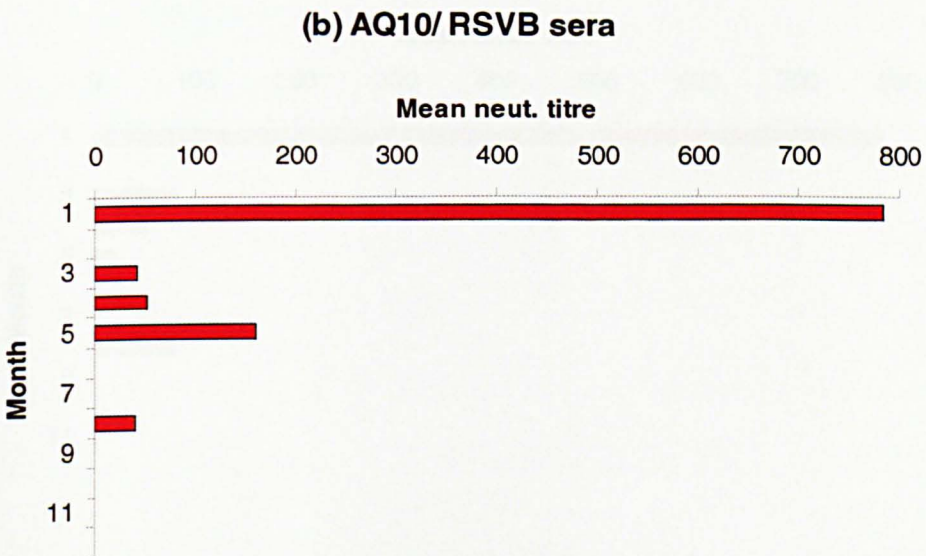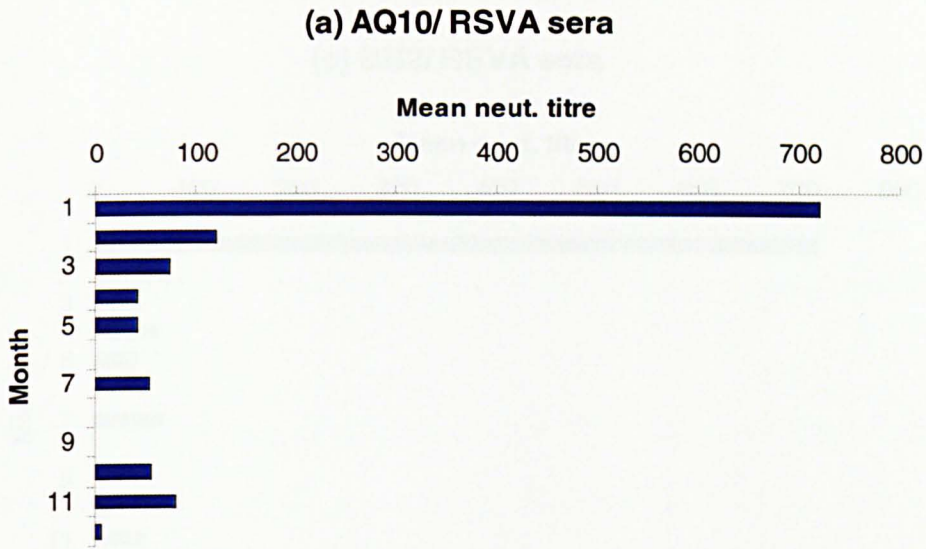
Figure 6.5:(a, b, c, d): Comparison of RSV A & B neutralization antibody titre in individual Qatari children acute serum samples from children challenged with AQ10 strain (a & c) or challenged with BN2 strain (b & d).

(c) BN2/ RSVA sera

(d) BN2/ RSVB sera
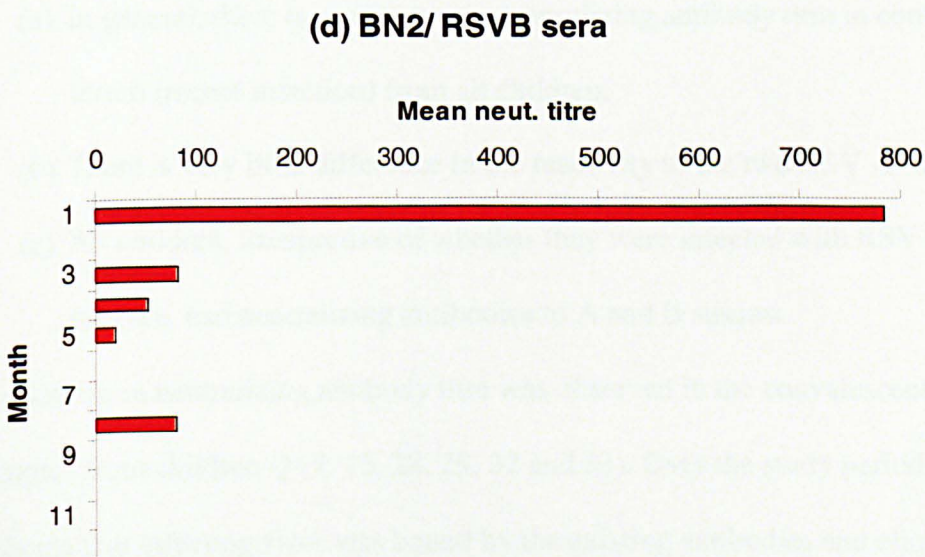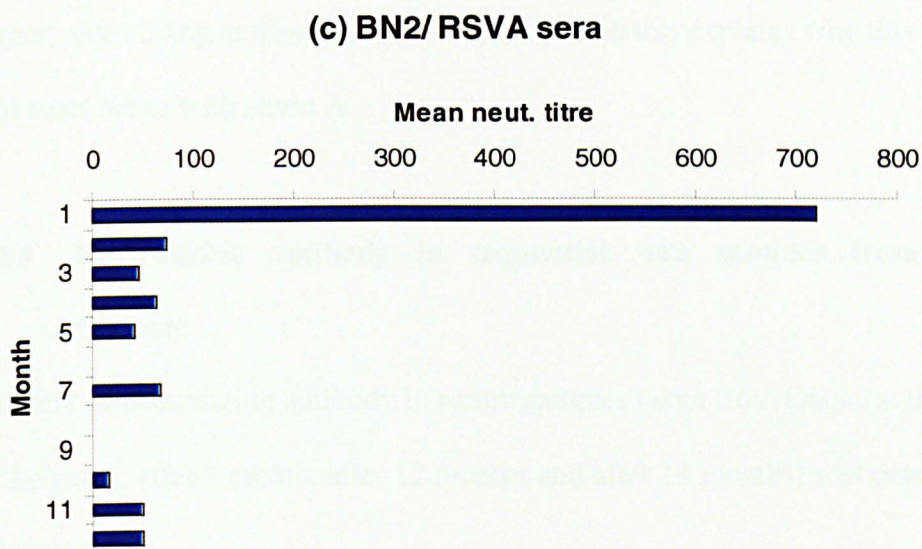
Each data point represents a serum sample of Qatar children serum above 1 month of age while less than 1 month represent neonatal sera (N= 20), all these collected at the same collecting time.

The question arises as to whether neonatal sera with high titres of neutralizing antibody to RSV A also react with a high neutralizing antibody titre to RSV B. Results in **Figure 6.6.b** show that this group of children were born with slightly higher neutralizing antibody titres to A than B, and this explains why this group of sera react better with strain A.

6.2.6 Neutralizing antibody in sequential sera samples from individual children:

The titre of neutralizing antibody in serum samples taken from Qatar (at the beginning of the study, after 1 month, after 12 months and after 13 months) was determined (**Figure 6.7**).

The following points can be drawn from these results: -

(a) In general, there is an increase in neutralizing antibody titre in convalescent serum (recent infection) from all children;

(b) There is very little difference in the reactivity to the two RSV A strains;

(c) All children, irrespective of whether they were infected with RSV A or B subtype, had neutralizing antibodies to A and B strains;

A decrease in neutralizing antibody titre was observed in the convalescent serum samples from children Q (7, 15, 28, 29, 32 and 33). Over the study period this could indicate that infecting virus was bound by the existing antibodies and eliminated but this did not induce a boost in antibody titre. The failure to detect virus after re-infection may be because the shedding period is usually very short in secondary infections due to the presence of preexisting antibodies; and

Samples from children Q (13, 17, 18, 19, 21 and 34) show an increase in the titre of neutralizing antibody over the study period, indicating a recent infection with RSV.

Figure 6. 7: (a, b, c): Neutralizing antibody titre in sequential samples from Qatar children (a) against RSV A A2; (b) RSV A/Q/10 and (c) RSV B N2.

(a)
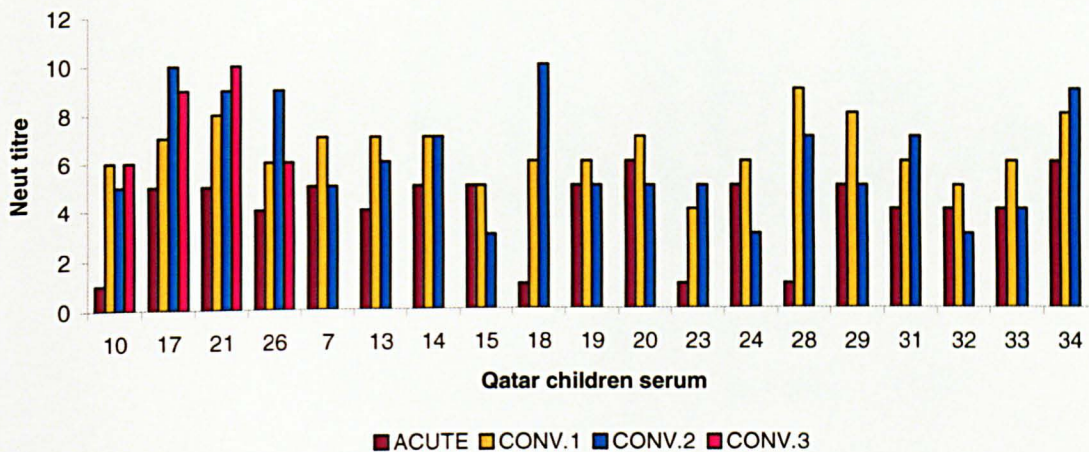
(b)
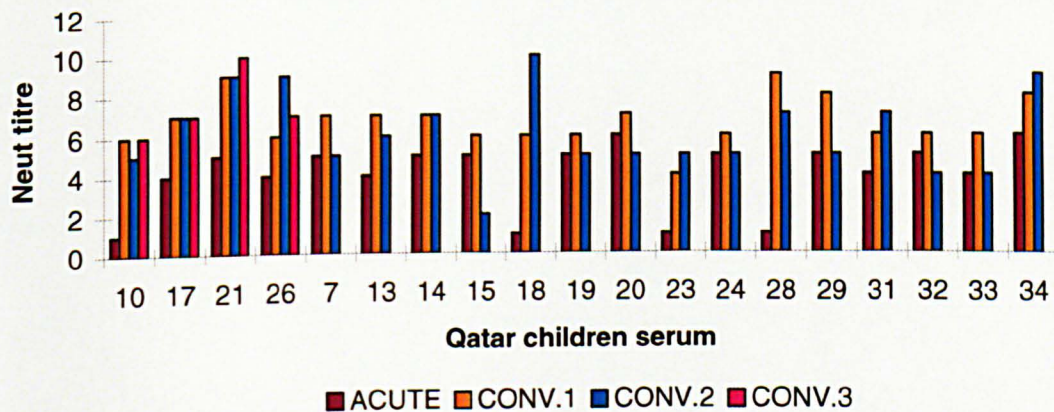
Figure 6. 7: (a, b, c): Neutralizing antibody titre in sequential samples from Qatar children (a) against RSV A A2; (b) RSV A/Q/10 and (c) RSV B N2.

C)
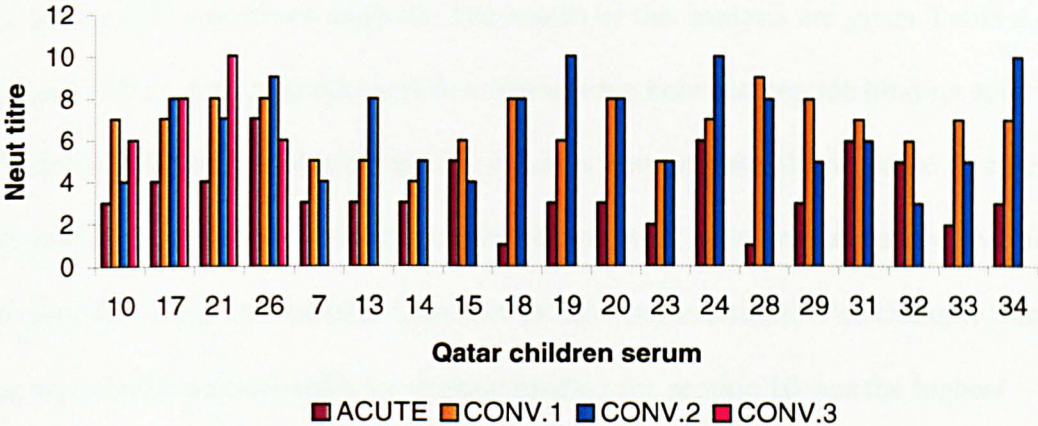
The neutralizing antibody dilution level as following:
1 = 1/5, 2 = 1/10, 3 = 1/20, 4 = 1/40, 5 = 1/80, 6 = 1/160, 7 = 1/320, 8 = 1/640, 9 = 1/1280, 10 = 1/2560.

6.2.7 Relationship between neutralizing antibody titre and peptide binding reactivity in Qatar sera:

To determine whether there was any correlation between neutralizing antibody titre and serum reactivity with the peptides in acute and convalescent sera, peptide binding values (OD) and neutralization titres using RSV strains (A (A/Q/10/00) & B (B/N2) were assessed by regression analysis. The results of this analysis are given **Table 6.4**. The results show that although there is a relationship between peptide binding activity (OD) and neutralizing antibody titre, the r-values were in general low, some r-values were negative (when one variable decreases as the other increases) and some r-values were positive (when one variable increases as the other increases). For example, the r-value with B/N2 neutralization vs. peptide binding for peptide 10 was the highest estimated (0.4) and r-values with A/10 neutralization vs. peptide binding for peptides 16, 14, 7 were also moderate (0.25, 0.23 and 0.22). The overall conclusion to be drawn from these analyses is that there was only a weak correlation between neutralizing antibody levels and reactivity with most sera of the peptides.

In general, the reactivity of antibodies to peptides was not correlates with serum neutralizing antibody.

6.2.8 Statistical analysis

In an attempt to do more statistical analysis on the relationship between the titre of neutralizing antibodies and peptide reactivity, descriptive statistical analysis was undertaken. The mean values of neutralizing antibody titre of serum samples were taken from children before and following the infection and tested with 2 RSV strains (RSV A/Q/10 and RSV BN2) and compared with mean values of peptide reactivity (OD). A comparison made between those sera had a greater rise in mean anti-RSV A

and B titre when collected after the infection (317 and 351) than before infection (54.8 and 55). Mean OD values were slightly increased in line with an increase of mean of neutralizing titres of A&B with peptides (2,3,10,11,13 & 14), there was a reverse correlation with peptide (1,7,8,12) and no difference with remaining of peptides **(Figure 6.8)**.

6.8.2. Further statistical analysis was performed using stepwise regression (taking peptide 14 as base line for the analysis), to study peptide reactivity in post infection sera (OD post). These sera have higher neutralization antibody titres than pre infection sera (OD pre). There is statistical evidence that an increase in neutralizing antibody titre is associated with increase in peptide 14 recognition by sera from children after infection particularly if the sera show reactivity with this peptide before infection **(Table 6.4** and **Figure 6.9 as an example)**.

In general it can be concluded that there is either no correlation or a weak correlation between neutralization activity and peptide reactivity (peptide 14) **(Figure 6.10** and **6.11)**

Table 6.4: Regression values (r) of peptide reactivity and neutralizing antibody of Qatari sera.

Peptide	r values of Neut (A/10) pre	r values of Neut (A/10) post	r values of Neut (B/N2) pre	r values of Neut (B/N2) post
1	0.085	**0.17**	0.169	0.111
2	0.079	0.062	0.049	0.004
3	0.005	0.016	0.046	0.062
4	0.026	**0.1722**	0.151	0.119
5	0.33	0.228	0.171	0.174
6	0.14	0.096	0.194	0.17
7	0.041	**0.215**	0.101	0.07
8	0.041	0.097	0.115	0.008
9	0.171	0.023	0.046	**0.187**
10	0.205	0.168	0.075	**0.404**
11	0.059	**0.106**	0.194	0.15
12	0.066	0.021	0.16	0.067
13	0.266	0.212	0.094	0.004
14	0.126	**0.228**	0.126	0.056
15	0.092	**0.2**	0.142	0.082
16	0.138	**0.253**	0.374	0.168
17	0.257	0.189	0.069	0.053

Red values indicate a positive relationship when neutralizing antibody reactivity increases as peptide reactivity increases after infection.

Figure 6. 9: (a & b): Statistical analysis was undertaken on data from mean values of peptide reactivity (OD) with Qatar children serum samples (pre & post infection).

Mean neut. (pre)= 55

Mean neut. (post)= 351
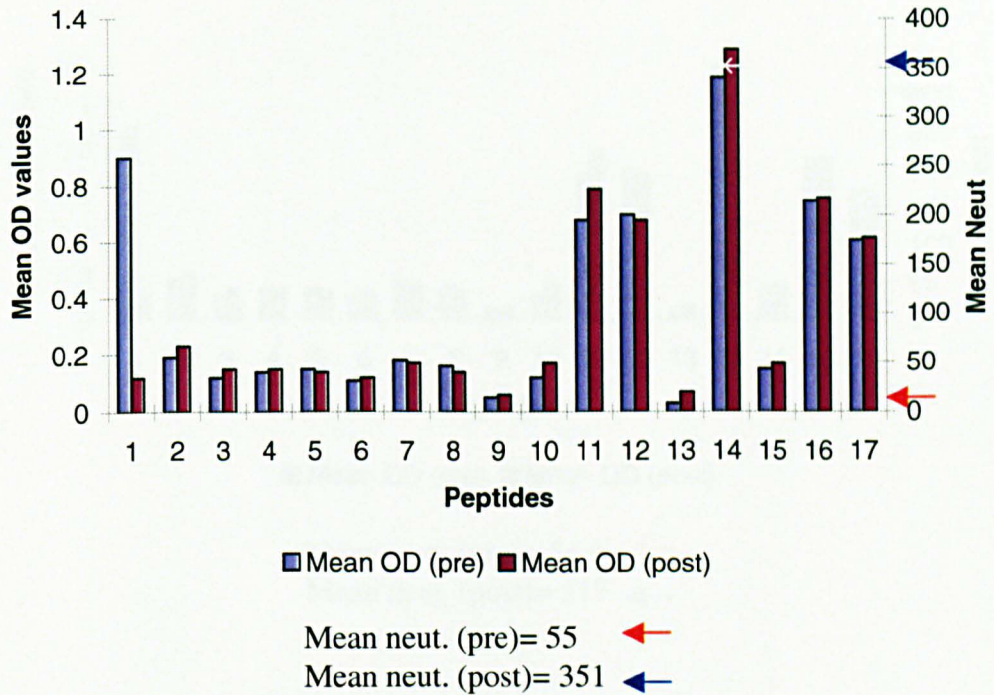
(a): Relation ship of neutralization titre and peptide binding reactivity (B/N2)

Figure 6. B:(a & b): Statistical analysis was undertaken on data from mean values of peptide reactivity (OD) with Qatar children serum samples (pre & post infection).
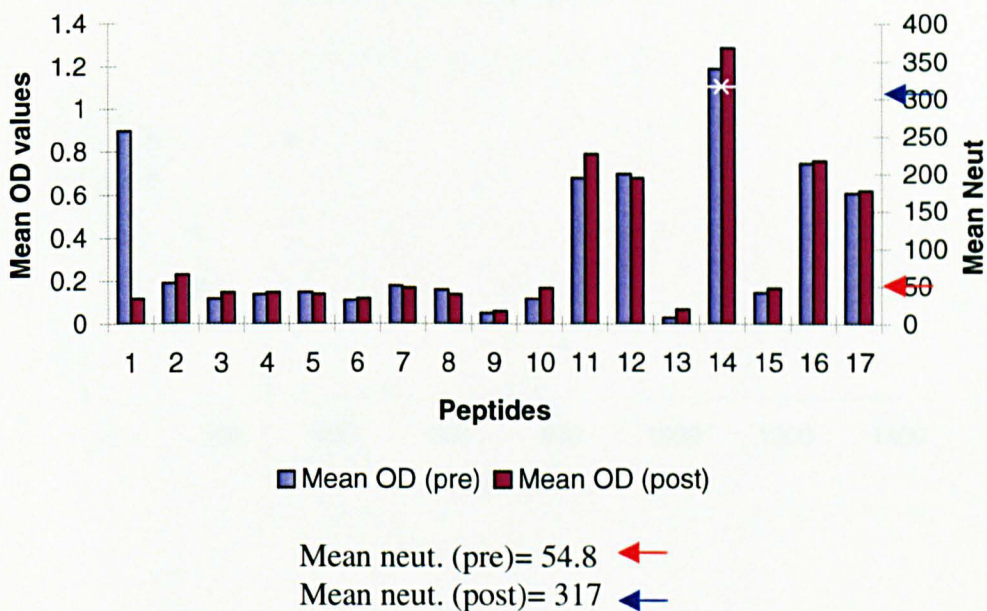
(b): Relation ship of neutralization titre and peptide binding reactivity (A/Q/10/00).

Comparing those that had a greater rise in mean anti-RSV titer (Mean neut. Post A&B) 317.03 & 351.721 with those that had lower mean anti-RSV titer (Mean neut. Pre A&B) 54.84 & 55.

The overall level of mean OD values were slightly increased with the increase of mean of neut. Titer of A&B with peptides (2,3,10,11,13 & 14), reverse correlation with peptide (1,7,8,12) and no difference with rest of peptides.

Figure 6. 9 : (a& b): Relationship of neutralizing antibody titre and solid phase binding to peptide 14 in all sera challenged with RSV A/10/00.

(a)

Pre RSVA sera/ peptide 14

Post RSVA sera / peptide 14

Figure 6. 9: (a& b): Relationship of neutralizing antibody titre and solid phase binding to peptide 14 in all sera challenged with RSV A/10/00.

(b)

Pre RSVB sera/ peptide 14

Post RSVB sera/ peptide 14
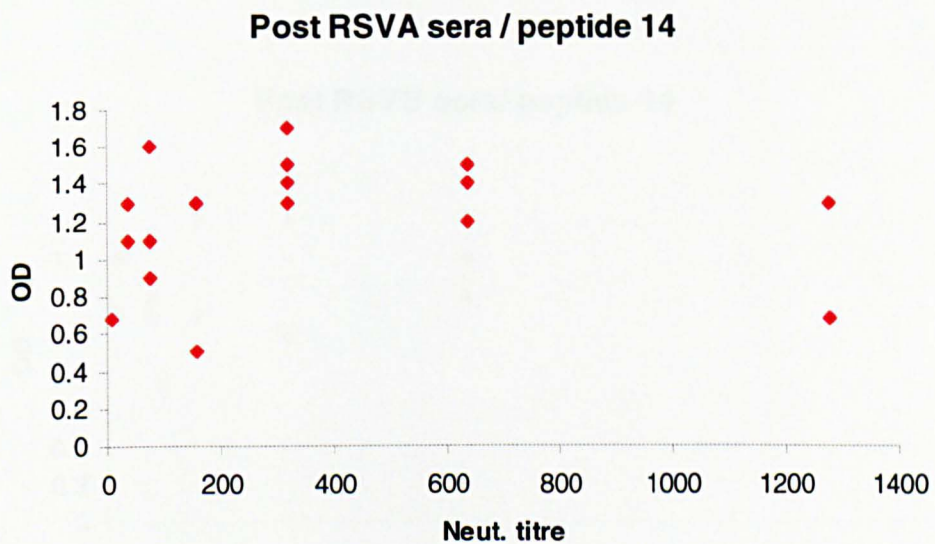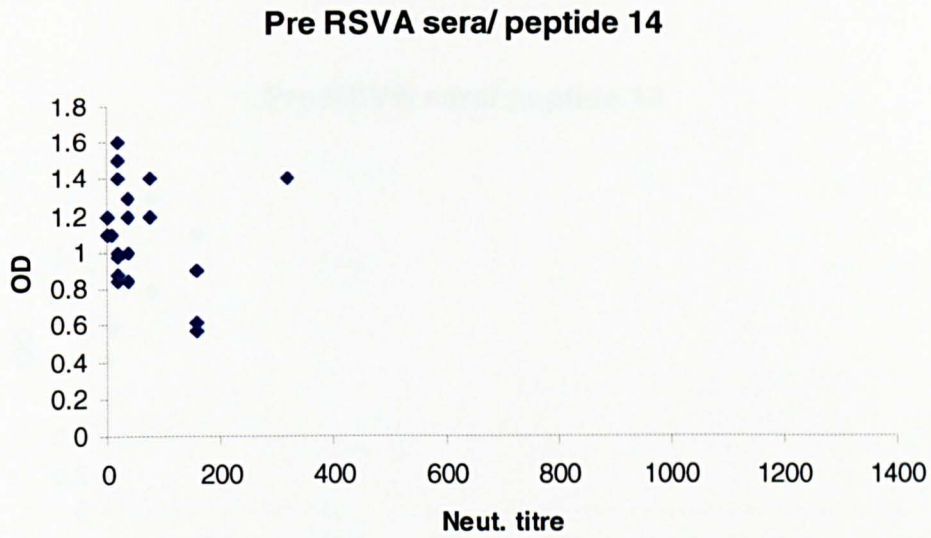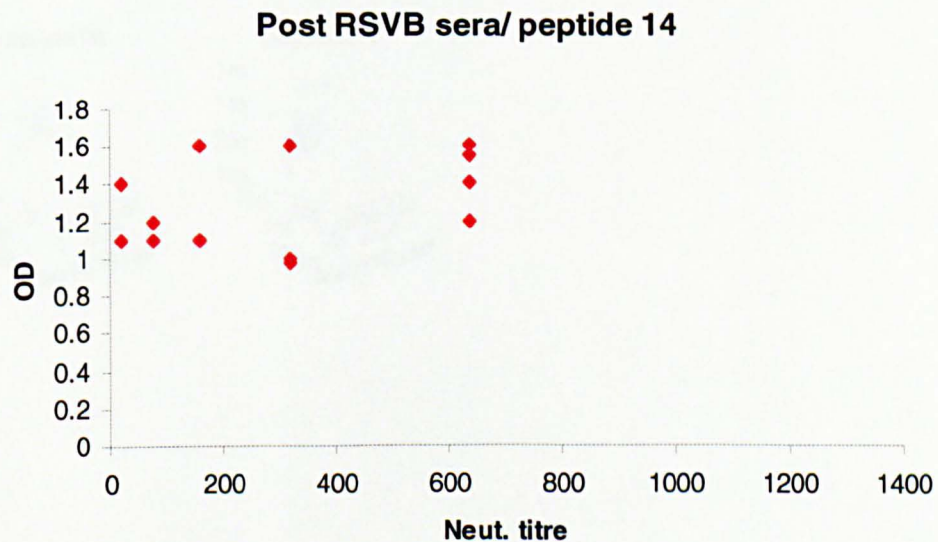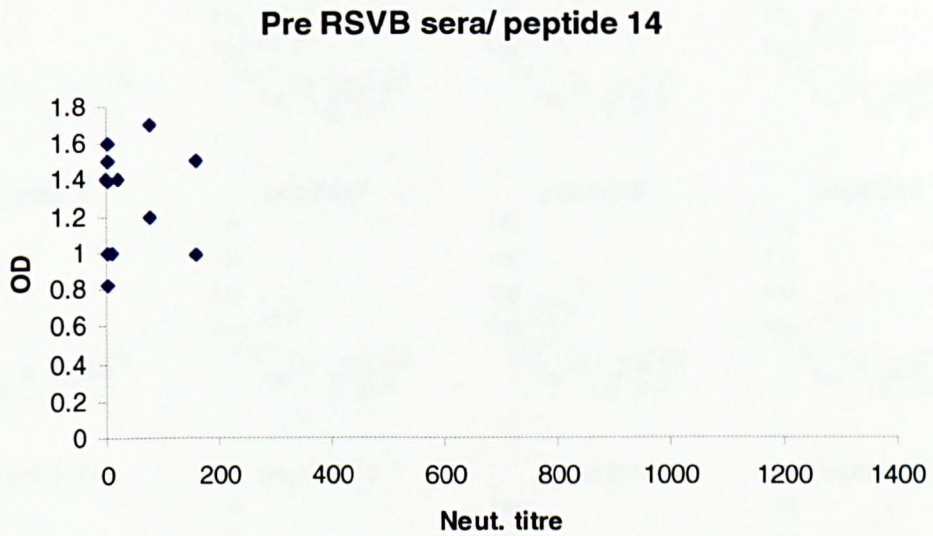
(a) Qatar children sera infected with RSV A strain and (b) Qatar children sera infected with RSV B strain.

Figure 6.18: Detection the correlation between peptide reactivity and neutralization titre
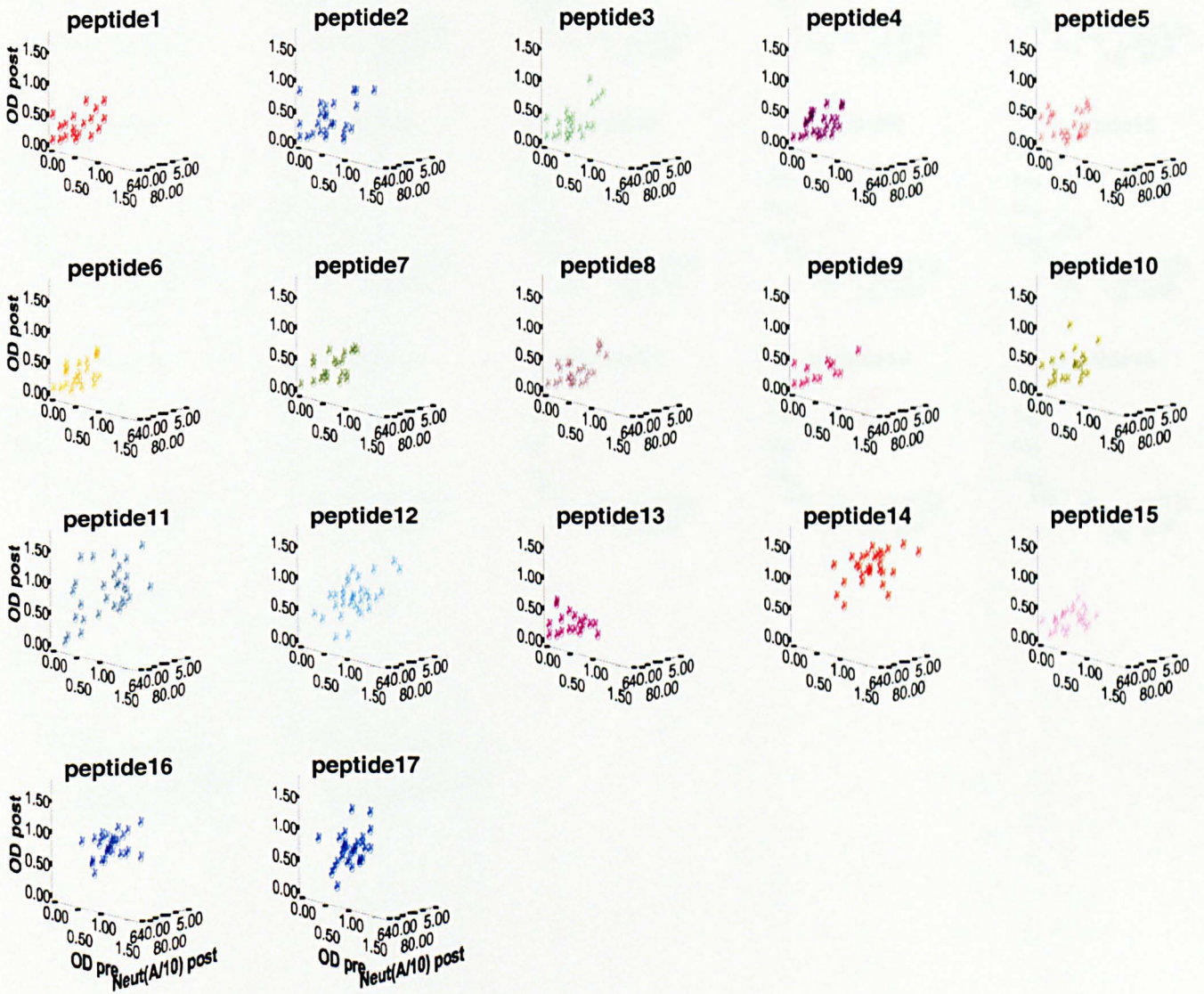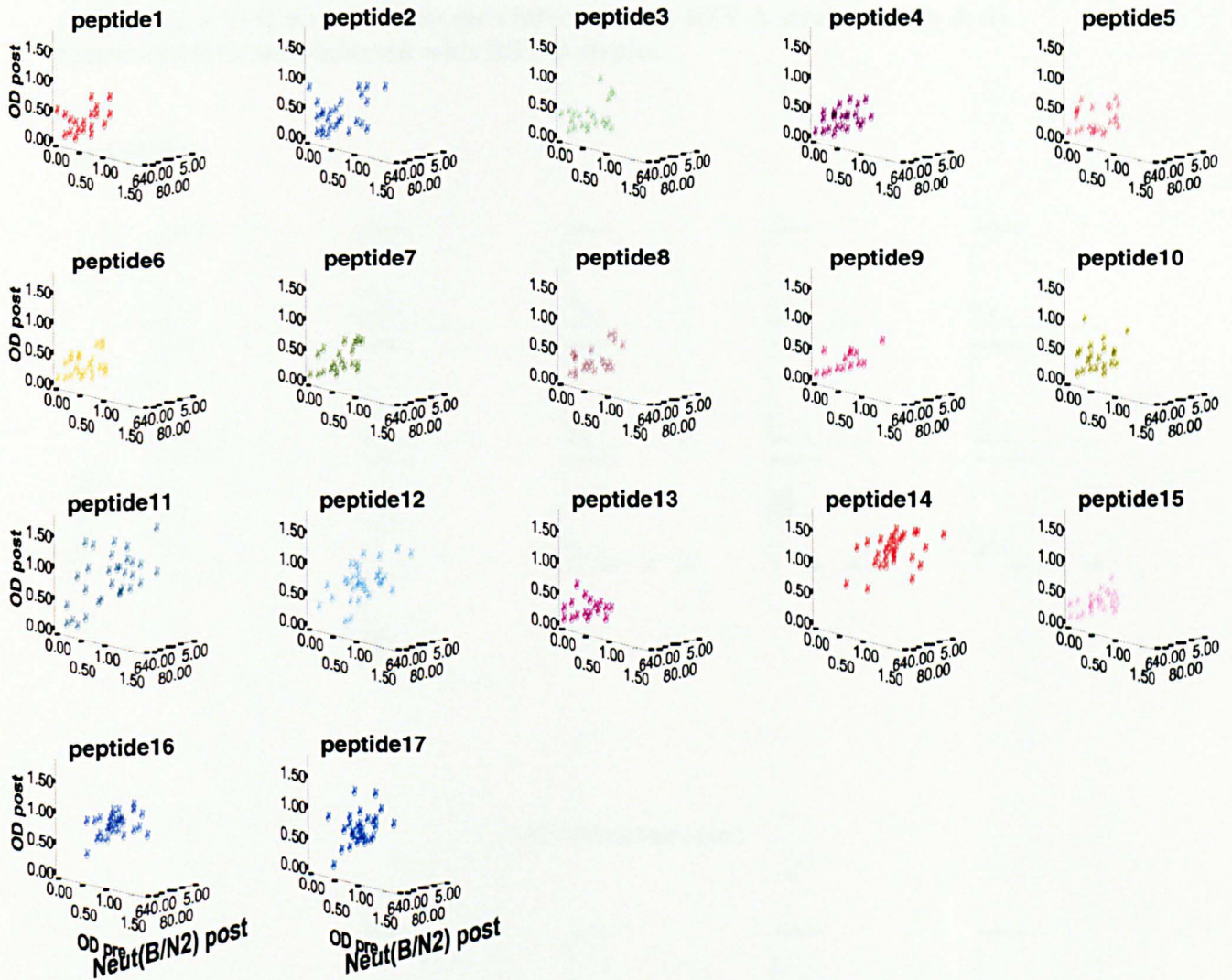
To detect the correlation between (peptide reactivity of post sera infection (OD post) with neut. Titer of the same group of sera in presence of peptide recognition with pre sera infection. There is statistical evidence that an increase in neut. titer is associated with increase of peptide recognition by post infection sera especially if sera react with this peptide before infection. We can see this relation clearly with shifting of points toward upper right especially with peptide 11 & 14

Figure 6.11:(a, b, c, d): Relationship of neutralizing antibody titre and peptide reactivity for all sera challenged with RSV A/10/00 (a & b) and RSV B N2 strains (c & d). (a & c) Qatar children sera infected with RSV A strain and (b & d) Qatar children sera infected with RSV B strain.

(a) AQ10A
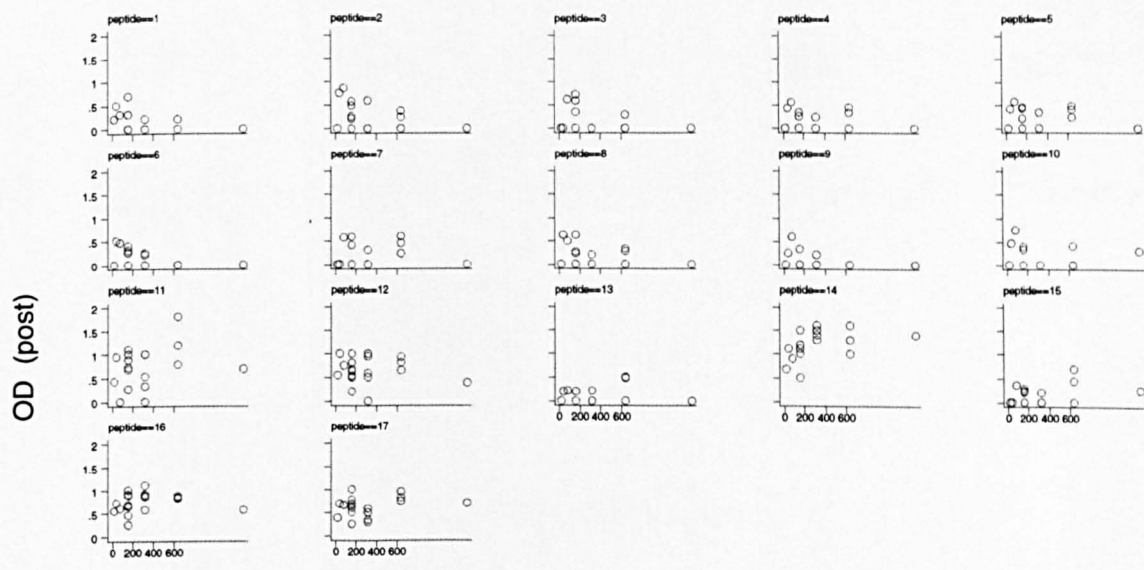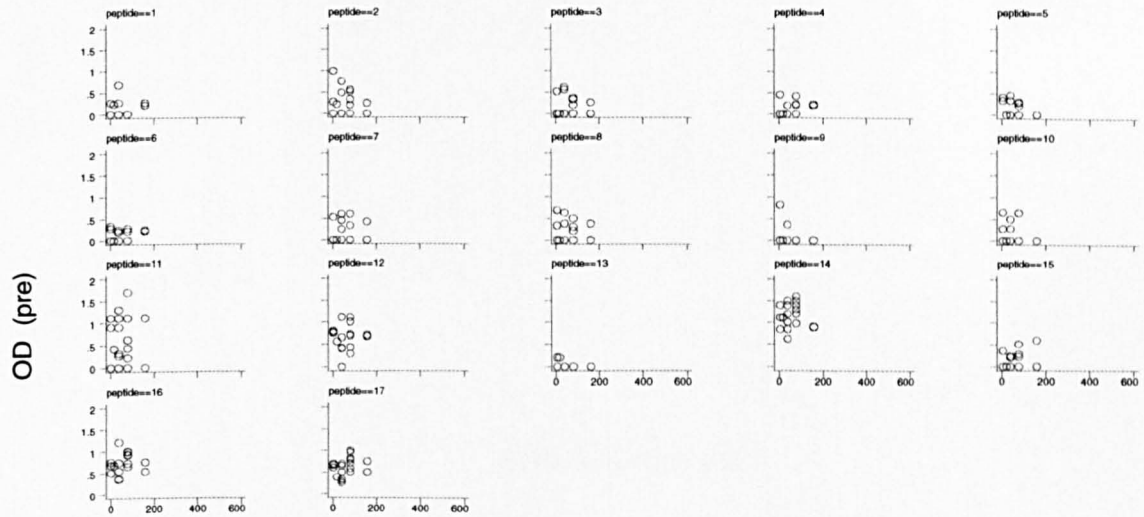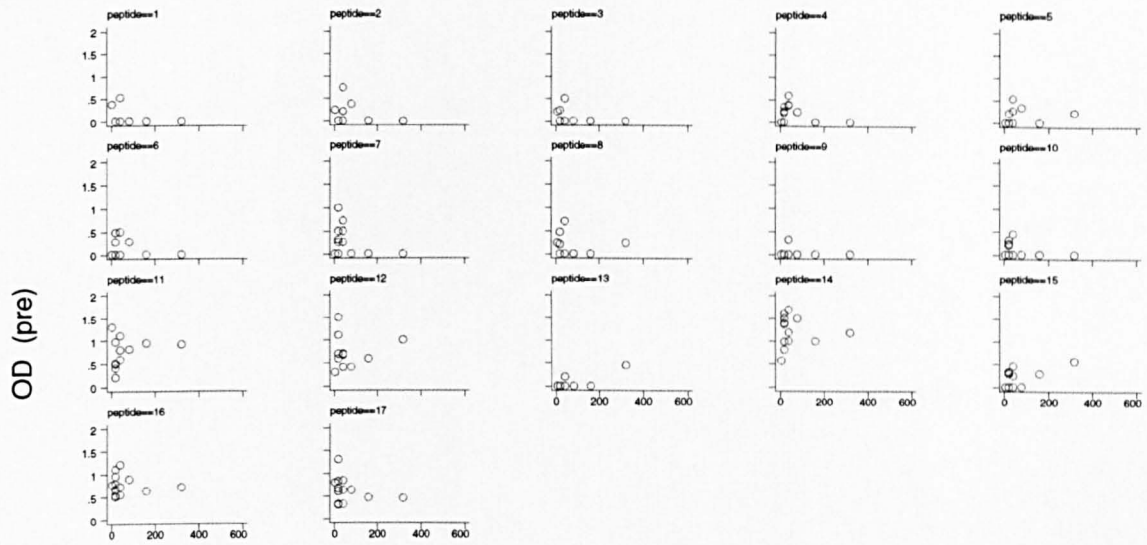
AQ10Neut.titer (pre)

AQ10Neut.titer (post)

(b) AQ10B

OD (pre)

AQ10Neut.titer (pre)

OD (post)

AQ10Neut.titer (post)

210

(c) BN2A

OD (pre)

peptide==1 peptide==2 peptide==3 peptide==4 peptide==5
peptide==6 peptide==7 peptide==8 peptide==9 peptide==10
peptide==11 peptide==12 peptide==13 peptide==14 peptide==15
peptide==16 peptide==17

BN2 Neut.titer (pre)

OD (post)

peptide==1 peptide==2 peptide==3 peptide==4 peptide==5
peptide==6 peptide==7 peptide==8 peptide==9 peptide==10
peptide==11 peptide==12 peptide==13 peptide==14 peptide==15
peptide==16 peptide==17

BN2 Neut.titer (post)

211

(d) BN2B

OD (pre)

BN2 Neut.titer (pre)

OD (post)

BN2 Neut.titer (post)

212
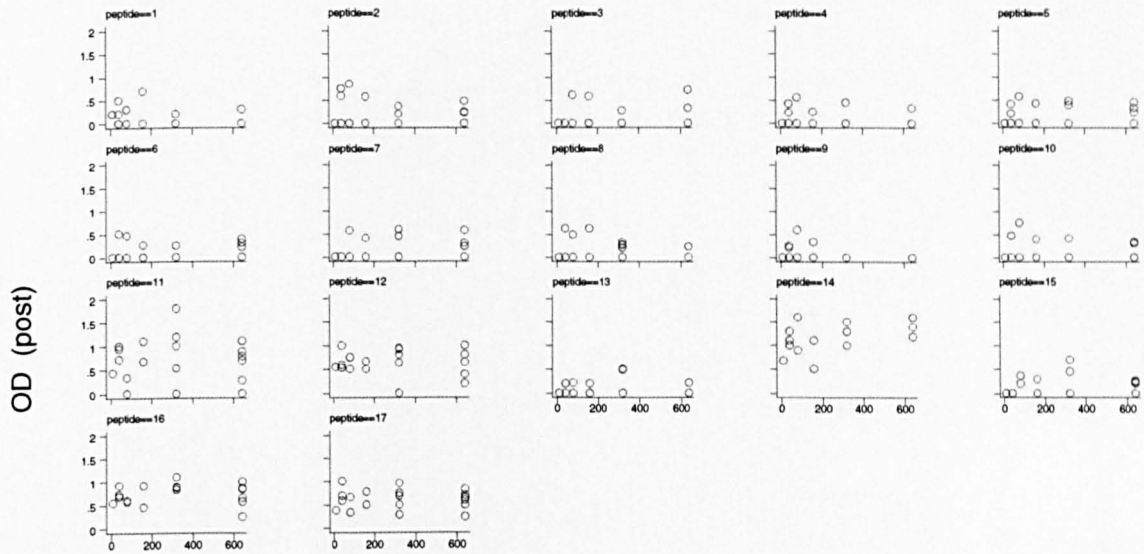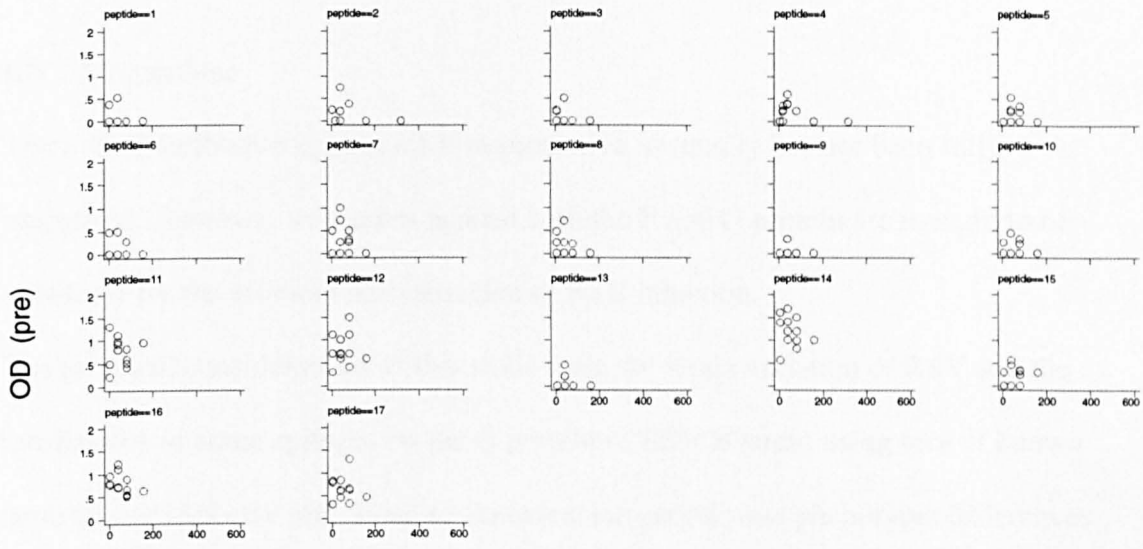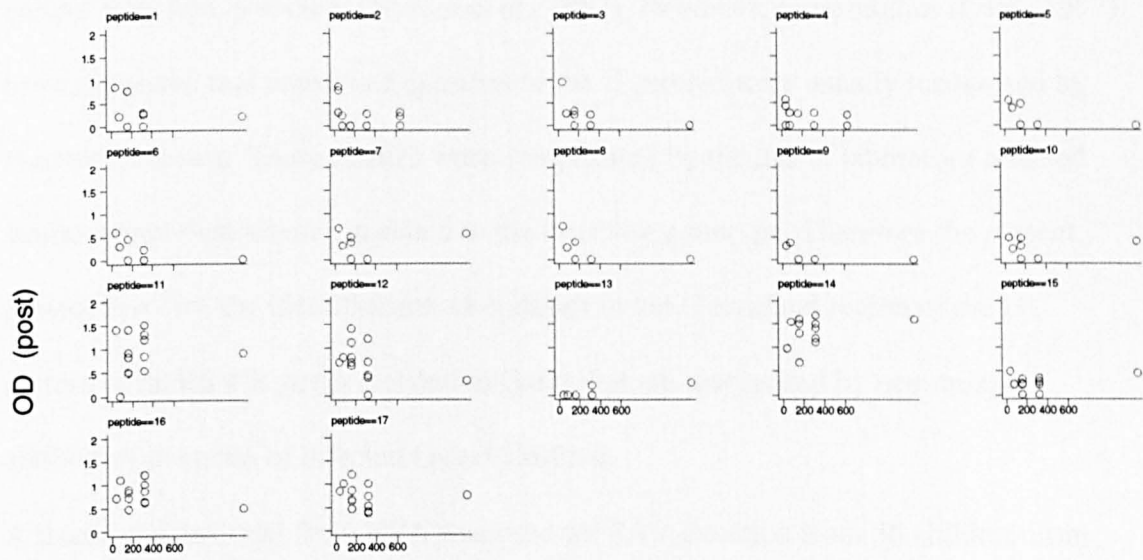
6.3 Discussion:

The role of antibodies against RSV in protective immunity has not been fully elucidated. However, antibodies against both the F and G protein are thought to be necessary for the efficient neutralization of RSV infection.

The principal considerations of this study were the strain variation of RSV and the localisation of some epitopes on the G protein of RSV B strain using sera of known neutralizing antibody titre. Despite the extensive genetic and phenotypic differences found in the expression of G protein epitopes among RSV isolates, there are also epitopes in the G protein that are conserved among viral strains (**Figure 5.2**). The relative dominance of different epitopes in humoral immune responses during a natural infection is unclear (Melero et al., 1997). However, some studies (Cane, 1997) have suggested that conserved epitopes of the G protein were usually recognised by convalescent sera. These studies were complicated by the use of laboratory-adapted strains rather than viruses matched to the infecting genotype. Therefore the present project involves the identification of epitopes in the C-terminal region of the G protein of an RSV B strain isolated in Qatar that are recognized by neutralizing antibodies in serum of infected Qatari children.

A fraction of material from NPA was used for RSV isolation from 36 children from whom paired sera were available from 2 seasons. In all of these children, respiratory material was positive by EIA and PCR, indicating a reasonable potential for recovery of RSV. 12/36 samples (33%) yielded RSV viruses. Among the isolates some viruses were easy to grow, giving 80-90% CPE after 3 days (e.g. A/Q/10/00 strain) and others required a longer incubation period and induced limited CPE, particularly RSV B strains. Others (Savon et al., 2000; Hall et al., 1990) have observed this phenomenon

with RSV B strain. Following multiple passage of virus through cell culture, the RSV

B and A strains could adapt to grow in the cell culture and hence lead to an increase

yield of virus and this has been carried out for one strain. The failure to recover the

RSV from previously identified positive samples (25 samples) was probably due to

the lag in time between receipt of specimen and attempts to isolate virus, which was at

least 6 months. Ideally, upon receipt of a sample, an aliquot should immediately be

placed in tissue culture, which should allow better recovery of the virus.

Electron micrographs obtained of RSV indicate different maturation stages of the

virus, from accumulation of the virus internal structure near the cell membrane to the

complete budding of the virus from this membrane. These photographs show two

types of budding virion (longitudinal and circular particles) as indicated in other

studies. These structural differences among Paramyxovirinae and Pneumovirinae

may have a bearing on the replication strategies or on their neucleocapside flexibility

employed by each virus subfamily (Bhella et al., 2002) and also show two ways for

the virus to leave the infected cell (either by budding or by lysis).

A neutralization assay was established for evaluation of paired sera from children, by

testing a panel of 14 primary anti RSV antibodies by the cell-based ELISA test (**Table

6.1**). It was found that some monoclonal antibodies were not useful for virus detection

in the neutralization assay, e.g. monoclonal antibodies raised against the nuclear

protein (NP) reacted well with RSV strain B in agreement with the results from

(Waris et al., 1990) but not with those from (Saville, 1997). In contrast, polyclonal

antibody AB 1128 goat anti RSV detected both RSV strains efficiently, an

observation also been made by (Martinez & Melero, 1998), but it was combined with

a lot of false positive due to cross reactivity. Positive controls were also prepared.

The categorization of RSV isolates into two major antigenic groups has led to interest in group-specific antigenic differences and their relevance to the epidemiology of RSV infection and illness. We examined antigenic group – specific serum antibody responses to 3 RS viruses (2 strains A & 1 strain B) in children who had been followed for 2 seasons. The aim was to pick up secondary infection but because secondary infections were milder, no obvious secondary infections were detected. Evidence for the prophylactic importance of the neutralization titre of anti RSV antibodies titre was demonstrated in the 1980s (Prince et al., 1985). A serum neutralization titre of 1:300 in cotton rats conferred almost complete protection. These data are compatible with the protective level of maternal RSV neutralizing antibodies in the neonatal group. This protective effect is more likely to be noted in the first months of life, and less so at or beyond 3 months of age, depending on the starting antibody concentration. Acute RSV infection in women during pregnancy is likely to boost maternal RSV antibody that can transmit transplacentally to the infant, providing protection early in life (Munoz & Glezen, 2003). Also, the presence of protective antibodies in the neonatal group and non protective antibodies in young children of Qatar study group, could be explained by that the fact that antibodies generated by young children after their first RSV infection are qualitatively different from passively-acquired maternal antibodies in an age-matched group of non-infected children (Roca et al., 2003). In addition, maternally-acquired antibodies are likely to have developed after several infections in the mothers who might be expected to have accumulated a cross-reactive pool of antibodies as well as more avid antibodies to cross-reactive epitopes.

If a protective neutralizing antibody titre (e.g. > 1:320) could be established, then screening of various target populations would provide an estimate of the number of

individuals at risk of infection. This information would be useful in targeting groups

for vaccination (Falsey, 1998).

Children who experienced primary infections with one strain A genotype made

significant levels of antibody cross – reactive with antigens of the other A genotype

and B strain in the virus neutralization assay and in the peptide binding assay. Also,

most children with primary infections with group B viruses had similar antibody

levels in the neutralization assay with A strain and similar levels of peptide reactivity.

Muelenaer et al. (Muelenaer et al., 1991) studied the antigenic group – specific serum

antibody responses to first and second infection. They observed significant cross –

neutralization of group B viruses by convalescent sera obtained from children who

had experienced group A infections, but not the converse. The present study showed

significant levels of cross reactivity in neutralizing antibodies and peptide reactivity in

serum from children infected with group A or B viruses and there is statistical

evidence that an increase in neutralization titre is associated with an increase of

peptide recognition by using same serum panel from children after infection

especially if these react with peptide before infection.

These findings suggest that at least part of the response in children was directed to

epitopes conserved between groups on the F and /or the conserved regions of the G

protein, it well be useful in future to measure how much of neutralizing antibodies

against G protein and how much against F protein among these sera. Also, using

peptides from the C-terminal of the G protein as antigens (Cane, 1997) showed that

infant responses can recognize strain-specific epitopes, although these may be

masked, in part, in the fully glycosylated protein (Palomo et al., 2000). Therefore, it is

reasonable to propose that the strain-specific element of the response is directed

against the G glycoprotein. That these antibodies were not detected in the neutralization test suggest that antibodies directed to hyper variable regions of G protein may have low neutralizing capacity as found in other work (Roca et al., 2003). Nevertheless, it is still less clear whether differences between strains within the same group affect susceptibility to infection. In animals, the titres of neutralizing antibody and the level of protection from virus challenge are not significantly different when strains from different genotype within the same group are used (Jones et al., 2002) and (Sullender et al., 1998) and this is consistent with the results presented here. On the other hand, the annual shift in the group or genotype of viruses causing outbreaks in communities suggests that differences between strains in the same group may affect susceptibility to infection (Cane et al., 1994; Hall et al., 1990; Peret et al, 1998). It is possible that a virus from a new genotype can transmit more efficiently or be more pathogenic because the RSV immunity in the population is most effective against a recently circulating genotype.

The observation that the peptide reactivity of an antiserum was not correlated with neutralization activity indicates that this approach is of limited value.

7 Chapter 7: General summary and Conclusions

RSV is a well-recognised cause of severe respiratory illness in infancy, in the elderly and in immunocompromised patients (Falsey et al., 1995; Raboni et al., 2003). Little is known about RSV infection in the Gulf region (Rajala et al., 2003). The conclusion of the work reported here was that RSV is responsible for high number of paediatric seasonal hospital admissions in Qatar (38%). This finding highlights the need for an effective RSV vaccine in children. Although the work presented here has revealed that RSV accounts for a significant proportion of illness presenting as disease of the respiratory system, there is still a large number of other diseases of the respiratory system remaining un-diagnosed. Investigation of the samples from which no virus was identified by PCR may suggest that other significant pathogens circulate within the community during the winter months e.g. the new paramyxovirus (the human metapneumovirus hMPV (van den Hoogen et al., 2001), that give an overlapping or similar clinical picture to that of RSV infection (Chen et al., 2004). Preliminary data suggest that hMPV may be responsible for approximately 7-8% of viral respiratory tract illnesses (RTI) in children (Stockton et al., 2002; Freymouth et al., 2003). Application of a multiplex PCR to detect targets other than RSV and influenza in these samples would thus be informative. Follow up of the patients would also be useful as the duration of the symptoms was not available for the work presented here. Several factors need to be monitored along with duration of the symptoms, including the measurement of cytokines, temperature, requirement for ventilation etc. It would only be possible, however, to conduct these types of studies volunteers or from patient's records after their permission or parent permission.

No attempt to monitor the circulation of RSV beyond the winter season was made here. Although hospital reports of RSV within HMC are very few during the summer,

the collection of samples from the paediatric department for analysis using the sensitive method of PCR may reveal background levels of circulation sporadic cases. In addition, little is known about RSV infection in the general community especially in elderly population in Qatar and the relationship to their admission to hospital. Expansion of the knowledge of RSV circulation and strain distribution within the general community will help with any vaccine design and delivery programme in this part of the world.

For studies where the samples are of poor quality and low virus copy number, the storage of specimens can be critical. For maximum recovery of an RNA virus the stability of the viral RNA in transport media may be crucial. It would be better for all of the samples to be analysed upon receipt, preferably with virus grown to high titre in tissue culture. This is not always possible, however. Obviously, the longer the time the virus remains in sub-optimal conditions, the lower recovery of that virus. Steps to improve subsequent detection of virus after specimen receipt may include preserving the sample into guanidinium- based buffers before long storage, by collection in guanidinium stabilization buffer, binding matrix glass fibre fleece (or on filter paper impregnated with guanidinium buffer) and elution in low volume or store the sample as a primary PCR product after the transcription step (cDNA genome which is more stable than RNA genome) (DeGraves et al., 2003).

It has been demonstrated in this work that RSV infection in Qatar is highly seasonal, positively correlated with relative humidity and rainfall and inversely correlated with temperature. This type of information could be helpful for healthcare workers, when they intend to use the available anti-viral drugs or treatments of RSV (e.g.palivizumab

and aerosolised ribavirin). However, with several new antiviral compounds active against RSV now in the early clinical stages of development, targeted treatment of illness might become a realistic option. New anti-RSV agents would need to be tested in young adults and the elderly, and targeted treatment would need appropriate tests for the rapid diagnosis of RSV. In children, the titre of RSV in the nasal secretions is high, so rapid diagnosis is easy with either the indirect fluorescent-antibody test or commercial EIA (Zambon et al., 2001).

In addition, studies of the role of other environmental factors (e.g. environmental pollutant; diesel engine emission etc (Harrod et al., 2003) and their effect on the RSV season would be of interest, e.g. Martinez (2003) suggests that both genetic and environmental factors determine the type of immune response to the acute RSV infection and that this response, in turn, may affect the development of the control mechanisms involved in the regulation of airway tone. Factors associated with increased risk are considered to be mostly environmental in nature (Munoz & Glezen, 2003), and especially social/ environmental conditions, as air quality could contribute to the high RSV morbidity reported by large American or European centres, mostly located in dense urban environment (Brandenburg et al., 1997).

The present genetic analysis of a recent RSV season in Qatar confirms the previously described higher variability found among group A isolates. Large differences were shown between group A and B isolates when a detailed comparison of different genetic features was performed. These differences may play role in the varied host immune response induced in infected children against the different RSV group. In addition, most of the Qatar RSV sequences analysed were genetically closely related

to previously describe viruses worldwide. The results therefore provide additional insight into the genetic and antigenic structure of both groups of RSV co-circulating in Middle East region and will contribute to the overall understanding of RSV circulation in the world. Also, it is still important to know whether the general community, especially the adult community, is infected with the same viral strains as infants and children. This is because any vaccine, for RSV, will ultimately be aimed at the infant and children populations (DeVincenzo, 1997). For example, if the viral strains circulating in the general community are distinct from those seen in the hospitalised infant population, they may represent a potential pool from which infants and children could be re-infected, post vaccination.

The G protein gene displays the highest capacity for differentiating between RSV strains (Simoes, 2001). Extensive analysis of RSV G protein variability has been performed with isolates from antigenic group A (Cane & Pringle, 1991; Cane & Pringle, 1995a; Garcia et al., 1994; Thompson et al., 2003). Human antibody responses to linear epitopes of the variable region of the group B RS virus G protein analysed here have been assessed. The reactivity of human antibodies with synthetic peptides varied with the infecting RS virus group. In addition, all of the defined linear epitopes involved potential N-linked glycosylation sites in some of the RSV virus isolates. It has been suggested that the modulation of glycosylation sites might be a mechanism for evasion of the host immune response by RS viruses (Anderson et al., 1991). The variable reactivity of human antibodies against peptides from this region of the G protein suggests the potential importance of antigenic changes in this region (peptide 1 to 10). Other workers have suggested that the final 68 C-terminal amino acids do not contribute to the major epitopes(s) of the G protein that are involved in

inducing protective immunity (Olmsted et al., 1989). The work presented here has demonstrated a lack of correlation between peptide reactivity and neutralizing antibody titre for both strain A and B of RSV.

Contrary to what was observed in group B primary RSV infection, where cross-reactive antibodies were detected in only a minor proportion of children, antibodies recognizing epitopes of the G protein of group B viruses were present in children with group A primary RSV infection (Hendry et al., 1988). Based on the data presented here, and accepting that the three-dimensional structure of the G protein remains to be elucidated, it could be hypothesized that the different pattern and number of putative glycosylation sites at the C-terminal of the G molecule found among both groups of viruses (especially for RSV A strains) and different G protein length (or stop codon termination for RSV B strains) might explain previous observations, since extensive glycosylation of group A strains may help to mask epitopes that are also present in B strains, and because no information available from this work about the correct three-dimensional structure of the cysteine cluster, the suggestive receptor binding site (Melero et al., 1997). Thus, the present work doesn't exclude the possibility that C-terminal end of the RSV G-protein directly contributes to the complete version of G-protein epitopes.

The poor quality of antibody responses to essential viral configurations is one factor of a variety of factors interfere with antibodies production quality, natural infections sometimes elicit very poor functional antibodies responses (e.g. HIV, Ebola virus and Dengue virus) (Burton & Parren, 2000).

Early studies described a " nonneutralizable" fraction of virus, which persisted even at high antibody concentrations. These nonneutralizable fractions have been described

for hepatitis A and hepatitis C as a result of virus association with lipids or lipoproteins (Burton et al., 2000). Also, some nonneutralizing antibodies arose because of a failure to appreciate that antibodies that bind to isolated envelope molecules do not necessarily, and very often do not, bind to envelope spikes. A classic example is HIV-1 where many antibodies have been described which bind with high affinity to monomeric gp120 or unprocessed gp 160, very few of these however showing substantial affinity for envelope spikes (Parren et al., 1998). The response to HIV-1 may be somewhat more complex than indicated, whereas immunogenicity may be low for conserved regions of the envelope spike, it is much higher for the variable regions.

Therefore, antibody neutralization and binding to envelope spike (especially epitopes located at C-terminal end of the RSV G protein) are very closely related, and any change at this part of molecule by adding, removing, glycosylation changes or even a single amino acid substitution may trigger a conformational changes in the envelope, for example, favour fusion or such change may non-specifically reduce binding between virion and antibody molecule.

Testing this hypothesis could be done by using an antigen-presenting system (Buratti et al., 1998)that preserve epitope conformation in the investigation of their immune responses or by using neutralization escape mutant system (McLain et al., 2001) and constructed either the whole longest G protein or different length of carboxyl ends of RSV G protein and clone it in a vector, then raising antibodies against these protein and test it for the presence of any escape mutant.

The importance of strain variation in the induction of protective immunity is less clear. Only a limited number of amino acid changes are required to produce a resistant phenotype to neutralizing antibodies (Cane et al., 1994) or in other virus (e.g. HIV)

(McLain et al., 2001). Repeated infections are more likely to occur with a virus from the heterologous than homologous group, presumably because immunity is more effective against the homologous than against the heterologous group viruses. In addition, some strains may be more virulent than others (Anderson & Heilman, 1995). These data, plus the data indicating the existence of dual infections in the current study, suggest that a RSV vaccine should include strains from both groups A and B. An initial group of chimeric recombinant AB vaccines have been constructed (Whitehead et al., 1999) in which the F & G genes from wild-type (wt) strain A2 were removed and replaced with the F & G genes from strain B. The immunogenicity and efficacy of one particular AB chimeric vaccine candidate was assessed in chimpanzees and resulted in significantly less rhinorhea and cough than infection with wild-type RSV (Whitehead et al., 1999). Furthermore, in recent years, passive administration of anti-RSV antibodies to high-risk children has been found to effective in decreasing the chance of developing severe clinical disease upon RSV infection (Hemming et al., 1995), emphasizing the importance of neutralizing antibodies.

Protective antibodies against RSV are predominantly directed to the F & G proteins. Antibody responses to the F glycoprotein of RSV are cross – reactive with RSV strains, whereas antibody responses to the G glycoprotein are largely subgroup specific (Hendry et al., 1988). One explanation as to why some patients may have `breakthrough, RSV infection while receiving palivirzumab (humanized chimeric monoclonal antibody directed against the conserved neutralizing epitope of the F glycoprotein of the RSV) is that this type of treatment lacks antibodies against the G protein (Weisman, 2002). In addition, immunization work RSV B1/cp-52 indicated that responses to F alone were sufficient to mediate infectivity in vitro, albeit this viral

strain was markedly attenuated in vivo. Naturally occurring G protein deletion mutants have not been described and therefore it is reasonable to assume that G offers an advantage to the virus during infection in vivo (Feldman et al., 2001).

Any RSV subunit vaccine containing the G protein would require both A and B subgroup G protein to afford protection against viruses (Sullender et al., 1990). Using peptides representing antigen epitopes as sub-unit vaccines, it would be possible to avoid any epitope that is in any way responsible for pathology (e.g. the amino acid sequences of the G protein 184 – 198 that cause a typical pulmonary eosinophilia (Tebbey et al., 1998). Furthermore, the most immunogenic epitopes of the G protein from both strains can be used.

B-cell epitopes (e.g. peptide 14, a.a 150-165) that mapped to the central conserved domain were recognised by the majority of human sera used (<93%) in this study. However, other peptides from the central region that are predominantly recognised by human sera (e.g. peptides 16 and 17) needs to be studied further. Thus, structural features of the epitope, several host factors (i.e., antigen presentation, genetic back ground of the host) need to be studied since these can influence immune responsiveness and affinity and specificity of the antibody response (Partidos & Steward, 2002).

The variation in the G region was discussed earlier (Chapter 4) and any vaccine based on this region would need to take account of this variation. Vaccines based on the G protein of RSV are currently being developed, and any information on the extent of variation within this region (especially for RSV B strain) is therefore of use (Siegrist et al., 1999). The data presented in this study may contribute to overall understanding of the extent of variation in this region of the genome.

In addition to exposure environmental risk factors, other factors, such as serum neutralizing antibody titre and the individual's own immune response, may have a greater influence on disease severity than the nature of the infecting strain. Others have found that serum neutralizing antibody plays role in protecting an infant against RSV infection. The observation of Gelzen et al., (Glezen et al., 1981) and passive immunoprophylaxis studies in humans (Groothuis et al., 1993) show that a serum neutralizing antibody titre of 1:300 is sufficient to protect young infants against being hospitalized with RSV illness. Also, the anti-RSV antibody titre in pregnant women at the time of delivery was important in protecting their infants, Acute RSV infection in women during pregnancy is likely to boost maternal RSV antibody that can be transmitted transplacentally to the infant, providing protection early in life (Munoz & Glezen, 2003).

From the present study it was found that there was association between the presence of higher concentrations of maternal neutralizing antibodies to RSV and young age (>1 month), as others have found (Le Saux et al., 2003; Englund, 1994; Walsh & Falsey, 2004). The importance of the protection afforded by maternal antibodies is clearly seen in infants incapable of producing immunoglubulins are protected by maternal antibodies for the first 3 to 12 months after birth. Maternal IgG antibodies enter the fetal circulation through the placenta. Constant boosting of the immunety can be achieved by infections with various microbes. Examples are periodic reinfection by poliovirus, the persistence of low level of disabled measles virus, and controlled subclinical infection by persistent hepatitis B virus (HBV) (Zinkernagel, 2001; Rehermann et al., 1996).

Cross-reactivity of maternally transferred antibodies may be result of two different mechanisms, strain-specific immunity and strain-transcending immunity (or a

combination of both). First of all, it may be that multiple infections in the mother involving different virus strains results in a broad range of strain specific antibodies that are maternally acquired. Alternatively, the cross-reactivity of maternally transferred antibodies may be a consequence of high level of antibodies against conserved virus epitopes induced by repeated infection. There is no evidence from this study, however, that infants who developed RSV infection were deficient in strain-specific antibodies. But on the other hand, this study indicates that high levels of passively acquired antibodies may protect against RSV infection. It is likely, therefore, that boosting maternal antibodies may benefit infants during the first months of life. This type of data can be useful for maternal immunization programs against RSV.

In general, longitudinal community-based studies of RSV variability will be necessary to define precisely the contribution of antigenic variation or the amount or titre of neutralizing antibodies in RSV reinfection. This type of information will be important when we are planning the designing and timing of RSV vaccines, because it is likely that different RSV vaccines will be needed for the various populations at risk. For example, if we intend to use the vaccine in the beginning of each season for the target population (infant, elderly, pregnant women and immunocompromised patients) thus antigenic variation is more important and it will be important to up date the vaccine (especially epitope based vaccine) with protective epitopes from both strains of recent circulating strains for each year, as for the influenza vaccine (Simoes, 2003) and (Polack & Karron, 2004b). While if the amount and titre of the neutralizing antibodies are is more important, peptide based vaccine (non-replicating vaccine) that contains peptides from the conserved domains of both strains and both important glycoproteins

(F and G) for each season could be used. This would be particularly important if the season was characterised by high relative humidity, rainfall and low temperature.

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Appendix 1: Ethical Committee agreement

HAMAD MEDICAL CORPORATION   دولة قطر   مؤسسة حمد الطبية

Research Committee
Tel.392440
Fax.392402

AM.004D .2000   Date:02-04-99

To: Dr. Asma Al Thani

From: Dr. A.A. Gehani
         Chairman, Research Committee

Subject: "Study of Respiratory Syncytial Virus Infection in Healthy Children < 2 year Hospitalized in Qatar"

The committee had discussed and approved the above research, we would like to have an update of the research results.

Please don't hesitate to contact us should you need any further information.

Kind regards,

Cc. Dr.Ghalia Al Thani,MB,BCh,MRCP(UK)
Chairperson, Dept. Pediatric

Appendix 2: Stratagen Optiprime buffers compositions

Buffer No.	Tris-HCl	MgCl	KCl
1	100 mM pH 8.3	15 mM	250 mM
2	100 mM pH 8.3	15 mM	750 mM
3	100 mM pH 8.3	35 mM	250 mM
4	100 mM pH 8.3	35 mM	750 mM
5	100 mM pH 8.8	15 mM	250 mM
6	100 mM pH 8.8	15 mM	750 mM
7	100 mM pH 8.8	35 mM	250 mM
8	100 mM pH 8.8	35 mM	750 mM
9	100 mM pH 9.2	15 mM	250 mM
10	100 mM pH 9.2	15 mM	750 mM
11	100 mM pH 9.2	35 mM	250 mM
12	100 mM pH 9.2	35 mM	750 mM

Appendix 3: Qatar study samples

Patient No.	ACUTE	CONV.1	CONV.2	CONV.3	AGE/Month	TYPE	Isolate
Q10	Y	Y	Y	Y	9	A	A/Q/10/00
Q17	Y	Y	Y	Y	3	A	
Q21	Y	Y	Y	Y	3	A	A/Q/21/00
Q26	Y	Y	Y	Y	1	A	A/Q/26/00
Q7	Y	Y	Y		10	A	
Q13	Y	Y	Y		1	A	
Q14	Y	Y	Y		2	A	A/Q/14/00
Q5	Y	Y	Y		3	B	
Q18	Y	Y	Y		4	A	
Q19	Y	Y	Y		2	A	
Q20	Y	Y	Y		4	B	
Q23	Y	Y	Y		2	B	
Q24	Y	Y	Y		2	B	
Q28	Y	Y	Y		3	B	B/Q/28/00
Q29	Y	Y	Y		36	A	
Q31	Y	Y	Y		12	B	B/Q/31/00
Q32	Y	Y	Y		4	B	
Q33	Y	Y	Y		3	B	B/Q/33/00
Q34	Y	Y	Y		9	A	
Q1	Y	Y			24	A	
Q2	Y	Y			2	B	
Q4	Y	Y			2	A	
Q11	Y	Y			6	B	B/Q/11/00
Q16	Y	Y			3	A	
Q22	Y	Y			6	A	
Q27	Y	Y			11	A	A/Q/27/00
Q30	Y	Y			3	B	
Q35	Y	Y			9	B	
Q36	Y	Y			3	A	
Q37	Y	Y			10	A	A/Q/37/01
Q38	Y	Y			2	A	
Q39	Y	Y			7	A	A/Q/39/01
Q40	Y	Y			3	A	
Q41	Y	Y			3	A	A/Q/41/01
Q42	Y	Y			3	A	
Q43	Y	Y			22	B	

Y = Available isolate

Acute= at the beginning of the infection

Convalescent 1 = 1 month after the infection

Convalescent 2 = 1 year after the infection

Convalescent 3 = 1 year & 1 month after the infection

Qatar study samples; Age Distribution and type of RSV. Yellow rows indicate to availability of RSV A isolates while blue rows indicate to availability of RSV B isolates.

Appendix 4: Neonatal samples

Sera No.	AGE
44	1 D
45	4 D
46	1 D
47	1 D
48	1 D
49	2 D
50	1 D
51	4 D
52	1 D
53	2 D
54	1 D
55	1 D
56	1 D
57	2 D
58	1 D
59	3 M
60	29 D
61	1 D
62	2 D
63	7 D
64	1 D

D: Day
M: Month

Neonatal samples (Total number of samples and Age distribution). From children admitted to hospital for non respiratory system illness during the RSV infection season, and most of them are less than one month in age.

Appendix 5.a: The nucleotide sequence alignment of 10 Qatar RSV A strains

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              10        20        30        40        50        60        70        80        90       100       110
A2        : ATGTCCAAAAACAAGGACCAACGCACCACCAAGACACTAGAAAAAGACCTGGGACACTCTCAATCATCTATTATTCATATCATCGTGCTTATACAAGTTAAATCTTAAATC
A/Q/10/00 : .........C............G.............G...................T.................................................
A/Q/13/00 : .........C............G.............G...................T..........................................TT.T......
A/Q/14/00 : .........C............G.............G...................T..........................................TT.T......
A/Q/16/00 : .........C............G.............G...................T..........................................TT........
A/Q/17/00 : .........C............G.............G...................T..........................................TT.T......
A/Q/19/00 : .........C............G.............G...................T.................................................
A/Q/27/00 : .........C............G.............G...................T.................................................
A/Q/29/00 : .........C............G.............G...................T.................................................
A/Q/39/01 : .........C............G.............G.....................................................................
A/Q/41/01 : .........C............G.............G...................T.................................................


              120       130       140       150       160       170       180       190       200       210       220
A2        : TATAGCACAAATCACATTATCCATTCTGGCAATGATAATCTCAACTTCACTTATAATTGCAGCCATCATATTCATAGCCTCGGCAAACCACAAAGTCACACTAACAACTG
A/Q/10/00 : ...................T...T.................................................T..............................C.
A/Q/13/00 : ...................T...T...........................................................................
A/Q/14/00 : ...................T...T...........................................................................
A/Q/16/00 : ...................T...T...................................G.........................................
A/Q/17/00 : ...................T...T...........................................................................
A/Q/19/00 : ...................T...T...................................G.........................................
A/Q/27/00 : ...................T...T.........................................................................C.
A/Q/29/00 : ...................T...T.........................................T...............................C.
A/Q/39/01 : ...................T...T...........................................................................
A/Q/41/01 : ...................T...T...........................................................................


              230       240       250       260       270       280       290       300       310       320       330
A2        : CAATCATACAAGATGCAACAAGCCAGATCAAGAACACAACCCCAACATACCTCACCCAGAATCCCCAGCTTGGAATCAGCTTCTCCAATCTGTCTGAAACTACATCACAA
A/Q/10/00 : .........................A...................................G...............................C.........
A/Q/13/00 : .........................A...............................A.................................C.........
A/Q/14/00 : .........................A...............................A.................................C.........
A/Q/16/00 : .........................A.................................................................C.........
A/Q/17/00 : .........................A...............................A.................................C.........
A/Q/19/00 : .........................A.................................................................C.........
A/Q/27/00 : .........................A...................................G.............................C.........
A/Q/29/00 : .........................A...................................G.............................C.........
A/Q/39/01 : .........................A.................T...............................................C....T.....G...
A/Q/41/01 : .........................A.................T...............................................C....T.....G...


              340       350       360       370       380       390       400       410       420       430       440
A2        : ACCACCACCATACTAGCTTCAACAACACCAAGTGTCAAGTCAACCCTGCAATCCACAACAGTCAAGACCAAAAACACAACAACAACCAAAATACAACCCAGCAAGCCCAC
A/Q/10/00 : C...T...T...............CTG...T...T.CA...............T...............C....G.....T.....A.....
A/Q/13/00 : C..........G............CTG.......CA...............T...............C................A.....
A/Q/14/00 : C..........G............CTG.......CA...............T...............C....G.....T.....A.....
A/Q/16/00 : C...T...................CT........CA...............T...............C....G.....T.....A.....
A/Q/17/00 : C..........G............CTG.......CA...............T...............C................A.....
A/Q/19/00 : C...T...................CT........CA...............T...............C....G.....T.....A.....
A/Q/27/00 : C...T...T...............CTG...T...T.CA...............T...............C....G.....T.....A.....
A/Q/29/00 : C...T...T...............CTG...T...T.CA...............T...............C....G.....T.....A.....
A/Q/39/01 : C..........G............CTG.......CA...............T...............C................T.....A.....
A/Q/41/01 : C..........G............CTG.......CA...............T...............C................T.....A.....


              450       460       470       480       490       500       510       520       520       540       550
A2        : CACAAAACAACGCCAAAACAAACCACCAAACAAACCCAATAATGATTTTCACTTTGAAGTGTTCAACTTTGTACCTTGCAGCATATGCAGCAACAATCCAACCTGCTGGG
A/Q/10/00 : ...................................A.................T.......C.................................
A/Q/13/00 : ...................................A.................T.......C.................................
A/Q/14/00 : ...................................A.................T.......C.................................
A/Q/16/00 : ...................................A.................T.......C.................................
A/Q/17/00 : ...................................A.................T.......C.................................
A/Q/19/00 : ...................................A.................T.......C.................................
A/Q/27/00 : ...................................A.................T.......C.................................
A/Q/29/00 : ...................................A.................T.......C.................................
A/Q/39/01 : ...................................A.................T.......C.................................
A/Q/41/01 : ...................................A.................T.......C.................................


              560       570       580       590       600       610       620       630       640       650       660
A2        : CTATCTGTAAAAGAATACCAAACAAAAAACCTGGAAAGAAAACCACCACCAAGCCCACAAAAAAAACCAACCATCAAGACAACCAAAAAAGATCTCAAACCTCAAACCACA
A/Q/10/00 : .C.....C.................................................................G....C...............
A/Q/13/00 : .C.....C.................................................................G...C................
A/Q/14/00 : .C.....C.................................................................G....C...............
A/Q/16/00 : .C.....C....................................G...........................C...............
A/Q/17/00 : .C.....C.................................................................G....C...............
A/Q/19/00 : .C.....C....................................G...........................C...............
A/Q/27/00 : .C.....C.................................................................G....C...............
A/Q/29/00 : .C.....C.................................................................G...C................
A/Q/39/01 : .C.....C...............................................................C...............
A/Q/41/01 : .C.....C...............................................................C...............


              670       680       690       700       710       720       730       740       750       760       770
A2        : AAACCAAAGGAAGTACCTACCACCAAGCCCACAGAAAAGCCAACCATCAACACCACCAAAACAAACATCAGAACTACACTGCTCACCAACAATACCACAGGAAATCCAGA
A/Q/10/00 : .................TC..................................................A..................TC...C.......A.........
A/Q/13/00 : .................TC..................................................CC...C......A.........
A/Q/14/00 : .................TC..................................................CC...C.........
A/Q/16/00 : .................TC..................G..............................A...............CC...C.........
A/Q/17/00 : ..............CT.TC..................................................CC...C.........
A/Q/19/00 : .................TC..................G..............................A..................TC...C.......A.........
A/Q/27/00 : .................TC..................................................A..................TC...C.......A.........
```

```
A/Q/29/00 : ..............TC.....................................................A..................TC...C.......A.........
A/Q/39/01 : .........T..CT.TCT......................GT..............G.....G...........GA.......TC...C.................
A/Q/41/01 : .............TCT.......................GT..............G.....G...........GA.......TC...C.................


              780       790       800       810       820       830       840       850       860       870       880
A2        : ACACACAAGTCAAAAGGGAACCCTCCACTCAACCTCCTCCGATGGCAATCCAAGCCCTTCACAAGTCTATACAACATCCGAGTACCTATCACAACCTCCATCTCCATCCA
A/Q/10/00 : .................A.................A...T..A............................................T...T..........
A/Q/13/00 : .........C.......A.................A...T..A............................................T.............
A/Q/14/00 : .........C.......A.................A...T..A............................................T...T..........
A/Q/16/00 : .................A........T........A......A............................................T...T..........
A/Q/17/00 : .........C.......A.................A......A............................................T.............
A/Q/19/00 : .................A...........T.....A......A............................................T...T..........
A/Q/27/00 : .................A.................A...T..A............................................T...T..........
A/Q/29/00 : .................A.................A......A............................................T...T..........
A/Q/39/01 : .........C.......A.................A......A..................................C.....T.............
A/Q/41/01 : .........C.......A.................A......A............................................T.............


              890       900
A2        : ACACAACAAACCAGTAG
A/Q/10/00 : ..........ATGA---
A/Q/13/00 : ..........ATGA---
A/Q/14/00 : ..........ATGA---
A/Q/16/00 : ..........ATGA---
A/Q/17/00 : ........G.ATGA---
A/Q/19/00 : ..........ATGA---
A/Q/27/00 : ..........ATGA---
A/Q/29/00 : ..........ATGA---
A/Q/39/01 : ...........TG....
A/Q/41/01 : ..........ATGA---
```

(a) The nucleotide sequence alignment of 10 Qatar RSV A strains, the start codon (ATG) at nucleotide position 1 and the stop codon at position 890 and 900.

(b): The nucleotide sequence alignment of 5 Qatar RSV B strains

```
                10        20        30        40        50        60        70        80        90        100       110
BN2    : GGGGCAAATGCAACCATGTCCAAACACAAGAATCAACGCACTGCCAGGACTCTAGAAAAGACCTGGGATACTCTCAATCATCTAATTGTAATATCCTCTTGTTTATACAG
B/Q/5/00  : ...........................................................................T.....................................A
B/Q/11/00 : ...........................................................................T......................................
B/Q/18/00 : ...........................................................................T......................................
B/Q/28/00 : ...........................................................................T.....................................A
B/Q/33/00 : ...........................................................................T......................................

                120       130       140       150       160       170       180       190       200       210       220
BN2    : ATTAAATTTAAAATCTATAGCACAAATAGCACTATCAGTTCTGGCAATGATAATCTCAACCTCTCTCATAATTGCAGCCATAATATTCATCATCTCTGCCAATCACAAAG
B/Q/5/00  : .....................................T..........................................................................
B/Q/11/00 : .....................................T..........................................................................
B/Q/18/00 : .....................................T..........................................................................
B/Q/28/00 : .....................................T..........................................................................
B/Q/33/00 : .....................................T..........................................................................

                230       240       250       260       270       280       290       300       310       320       330
BN2    : TTACACTAACAACGGTCACAGTTCAAACAATAAAAAACCACACTGAAAAAAAACATCACCACCTACCTTACTCAAGTCCCACCAGAAAGGGGTTAGCTCATCCAAACAACCT
B/Q/5/00  : ...........T....................................G............T..................T.....................C.........C
B/Q/11/00 : ...........T....................................G............T..................T.....................C.........C
B/Q/18/00 : ...........T....................................G............T..................T.....................C.........C
B/Q/28/00 : ...........T....................................G............T..................T.....................C.........C
B/Q/33/00 : ...........T....................................G............T..................T.....................C.........C

                340       350       360       370       380       390       400       410       420       430       440
BN2    : ACAACCACATCACCAATCCACACAAATTCAGCCACAACATCACCCAACACAAAGTCAGAAACACACCACACAACAGCACAAACCAAAGGCAGAACCACCACCTCAACACA
B/Q/5/00  : ........C.............C...........T......T..T.....A...........T.............................................TC.....
B/Q/11/00 : ........C.............C...........T......T..T.....A...........T......................G.....................TC.C....
B/Q/18/00 : ........C.............C...........T......T..T.....A...........T......................G.....................TC.C....
B/Q/28/00 : ........C.............C...........T......T..T.....A...........T.............................................TC.....
B/Q/33/00 : ........C.............C...........T......T..T.....A...........T......................G.....................TC.C....

                450       460       470       480       490       500       510       520       530       540       550
BN2    : GACCAACAAGCCGAGCACAAAACCACGCCTAAAAAAATCCACCAAAAAAAACCAAAAGATGATTACCATTTTGAAGTGTTCAACTTCGTTCCCTGTAGTATATGTGGCAACA
B/Q/5/00  : ..A...............................C....G.......................................................................
B/Q/11/00 : ......................................C....G...................................C...............................
B/Q/18/00 : ......A...............................C....G...................................................................
B/Q/28/00 : ..A...............................C....G.......................................................................
B/Q/33/00 : ......A...............................C....G...................................................................

                560       570       580       590       600       610       620       630       640       650       660
BN2    : ATCAACTTTGCAAATCCATCTGTAAAACAATACCAAGCAACAAACCAAAGAAGAAACCAACCATCAAACCCACAAACAAACCAACCACCAAAACCACAAACAAAAGAGAC
B/Q/5/00  : .......C.............C...................T.........A...........................C...............................
B/Q/11/00 : .......C.............C...................T.........A...........................C...............................
B/Q/18/00 : .......C...........................................A................A.........A.......T...G..A.....A...........
B/Q/28/00 : .......C...........................................A................A.........A.......T...G..A.....A...........
B/Q/33/00 : .......C...........................................A................A.........A.......T...G..A.....A...........

                670       680       690       700       710       720       730       740       750       760       770
BN2    : CCAAAAAACACCAGCCAAAACGACGAAAAAAGAAACTACCACCAACCCAACAAAAAAAACCAACCCTCACGACCACAGAAAGAGACACCAGCACCTCACAATCCACTGTGCT
B/Q/5/00  : ...........T.........CT.........G..C.............G..........C..A.........G....................G..............
B/Q/11/00 : ...........T.........CT.........G..C.............G..........C..A.........G....................G..............
B/Q/18/00 : .....................CT.........C...............G..G........C..A.....AG.......................G..............
B/Q/28/00 : .....................CT.........C...............G..G........C..A.....AG.......................G..............
B/Q/33/00 : .....................CT.........C...............G..G........C..A.....AG.......................G..............

                780       790       800       810       820       830       840       850       860       870       880
BN2    : CGACACAACCACATTAGAACACACAATCCAACAGCAATCCCTCCACTCAACCACCCCCGAAAACACACCCAACTCCACACAAACACCCACAGCATCCGAGCCCTCTACAT
B/Q/5/00  : ..........G...CG..G.....GG....................................................................C....
B/Q/11/00 : ..........G...CG..G.....GG....................................................................C....
B/Q/18/00 : ..........G...CG..G.....GG....................................................................C....
B/Q/28/00 : ..........G...CG..G.....GG....................................................................C....
B/Q/33/00 : ..........G...CG..G.....GG....................................................................C....

                890       900       910
BN2    : CAAATTCCACCCAAAATACCCAATCACATGCTTAGTT
B/Q/5/00  : .................AC..T.G...T.........--
B/Q/11/00 : .................AC..T.G..----------
B/Q/18/00 : .................CC..T.G--------------
B/Q/28/00 : T...............C.........G..A......
B/Q/33/00 : ................C.........G..A......
```

(b): The nucleotide sequence alignment of 5 Qatar RSV B strains, the start codon (ATG) at nucleotide position1 and the stop codon (TAG) at position 900 for 2 viruses and at 896 for the rest, the strictly conserved nucleotide sequences at both strain sequences are underlined.

Appendix 6: Accession numbers for sequences that have been derived from databases as following:

A-subtypes:

Korean's strains: AF193304, AF193305, AF193312, AF193313, AF193309, AF193320, AF193326 and AF193327.

USA's strains: AF065258, AF065410, AF065407, AF065405 and AF065408.

Spain's strains: Z33427, Z33428, Z33432, Z33431, Z33430 and Z33423.

UK's strains: X73350and X73354.

Uruguay's strain: AF248642

Mozambique strains: AF309661, AF309663, AF309664, AF309659 and AF309655.

B-subtypes:

Korean's strains: AF193337, AF193330, AF193333, AF193334, AF193335, AF193337, and AF193336.

USA's strains: AF065250, AF065251, AF233924, M73542 and M73541.

UK's strains: AJ290201, AJ290202, AJ290203, AJ290209, AJ290214, AJ290219and AJ290222.

Uruguay's strain: AF251555, AF249877 and AF251557.

Mozambique strains: AF309668, AF309677, AF309679, AF309676, AF309678 and AF309684.

Appendix 7: B-cells epitopes identified from G protein of RSV

Regions (Amino acid no.)	Amino acid sequences	Type of antigen	Source of sera	Virus	Main finding & References
174 – 188	SICSNNPTCWAICKR	Peptide	Rabbit, Mice & Human	A2	Identified linear B cell epitopes
144 – 168	SKPTTKQRQNKPPSK		Rabbit, Human		
84 – 98	KNTTPTWLTQNPQLG		Rabbit.		
94 – 108	NPQLGISPSNPSEIT		Rabbit.		
134 – 148	KNTTTTQTQPSKPTT		Rabbit.		
184 – 198	AICKRIPDKKPGKKT		Human		(Norrby et al., 1987)
194 – 208	PGKKTTTKPTKKPTL		Rabbit.		
224 - 238	EVPTTKPTEEPTINT		Rabbit.		
C-Terminal end	G230...PTTKPG G230...PTTKPCKTFELPRARVNT	Expressed protein	Rat	A2	C terminal do not contribute to the major epitopes (Olmsted et al., 1989)
Whole protein	CDNA placed in vaccina virus vector	Expressed protein	Cotton Rat	B(8/60)	Subunit vaccine requires both A&B subgroup G protein. (Sullender et al., 1990)

Regions (Amino acid no.)	Amino acid sequences	Type of antigen	Source of sera	Virus	Main finding & References
174-188	KNTTTQTQPSKPTTKQRQNKPPSKPNND FHFEVFNFVPCSICSNNPTCWAICKRIPD KKPGKK	Peptide A	Rabbit & human sera	A2	Antigenic reactivity sites depends on disulphide bond (Akerlind-Stopner et al., 1990)
174-188	KGRITSTQTNKPSTKSRSKNPPKKPKDD YHFEVFNFVPCSICGNNQLCKSICTIPSN KPKKK	B		B(CH18537)	
174-187	KNTTTQTQPSKPTTKQRQNKPPSKPNND FHFEVFNFVPCSICSNNPTCWAICKRIPD KKPGK	Peptide	BALB/Mice	A2	Importance of the 174-187 region for protection and the 144-159 region to induce neut. Antibodies (Trudel et al., 1991)
Restriction fragment exchanges among regions of A & B G protein.	Amino acid 1-173, 174-214 and 215-end	Expressed protein	Monoclonal antibodies from Mouse Polyclonal antibodies from Rabbit	A Long & A2 B (18537)	Each region has role in MAbs & neut. Activity. (Sullender, 1995)

Regions (Amino acid no.)	Amino acid sequences	Type of antigen	Source of sera	Virus	Main finding & References
C-terminal	Variable 84-85 C-terminal	Expressed protein	Human Acute & Convalescent	Circulating strain	Human immune response relate to infecting genotype. (Cane et al., 1996)
173-187	CSICSNNPTCWAICK	Recombinant bacteriophage	Mice	Long	Identified protective epitopes in mice. (Bastien et al., 1997)
171 –178 174-187 171 –178 174-187 Recombinant protein	VPCSICSNNPTCWAICK SICSNNPTCWAICK VPCSICGNNQLCKSICK SICGNNQLCKSICK 125-203	Peptide A B Expressed protein	Long 8/60(B) Long	BALB/c mice	The importance of 171-187 & disulfide region for reactivity. (Simard et al., 1997)
229 – 243 262 – 276 280 – 294 247 - 258	KPTEKPTINTTRTNI KETLHSTSPEGNPSP YTTEYPSQPPSPSN LLTTNTTGNPEY	Peptide	109 sera 47 sera 1 sera 2 sera	Clinical isolate A strain	Identified linear epitopes that involved in potential N-glycolysation sites (Cane, 1997)

Regions (Amino acid no.)	Amino acid sequences	Type of antigen	Source of sera	Virus	Main finding & References
Conserved & C-terminal region	Epitopes C793 & 021/G 021/19G & 021/G	Expressed protein & peptide	Mouse & Human	A	Identified 3 types of epitopes: conserved, group-specific & strain specific that mainly at C-terminal end. (Melero et al., 1997)
Linear B cells epitopes	152-103, 165-172, 171-187 two overlapping epitopes 196-204	Peptide BBG2Na	Human.	A Long	B-cell epitopes recognized by human. (Plotnicky-Gilquin et al., 1999)
Synthetic peptide vaccine	174 – 187	Peptide	Mouse	A Long & Bovine	Complete protection against natural pathogen by mucosaley delivered synthetic peptide. (Bastien et al., 1999)
Vaccine antigen produce in plant	174-187	Peptide	Mice	A Long	Protective immunity by epitope (174-187) in mice. (Belanger et al., 2000)

Regions (Amino acid no.)	Amino acid sequences	Type of antigen	Source of sera	Virus	Main finding & References
Intranasal immunization of mice	144-159, 190-203 & 171-188.	Peptide	Mice	A long	Partial protection by intranasal immunisation using three peptide from G protein in mice. (Cano et al., 2000) & (Power et al., 1997)
5 – linear B cells epitopes	152 – 163, 165 – 172, 171 – 187 – (2 over lapping epitopes) & 196 - 204	Peptide	Human		Identification multi B-cell epitopes. (Power et al., 2001)
BBG2Na 130...173…176...182…186...230	TVKTKNTTTTQTQPSKPTTKQRQNKPPN KPNNDFHFEVFNFVPCSICSNNPTCWAIC KRIPNKKPGKKTTTKPTKKPTFKTTKKD HKPQTTKPKEVPTTKPVD	Vector Exp.	Mice	A Long	Importance of BBGN2 in inducing protection in different population target. (Dagouassat et al., 2001)
CX3CC region of G (a.a 169-191)	182-186 (CWAIC)	Purified G	Rbt.	A(Wt)	G protein interaction with CX3CR1 play role in RSV infection. (Tripp et al., 2001)

Regions (Amino acid no.)	Amino acid sequences	Type of antigen	Source of sera	Virus	Main finding & References
Region responsible of [1]PBMC from patient with asthma	151 – 206	Peptide	Human	A2	The role of G protein in wheezing. (Hancock et al., 2001)

[1]PBMC: Peripheral blood mononuclear cells

.

Appendix 8: Amino acids abbreviations

Amino acid	Symbol	Abbreviations	Amino acid	Symbol	Abbreviations
Alanine	Ala	A	Leucine	Leu	L
Arginine	Arg	R	Lysine	Lys	K
Asparagine	Asn	N	Methionine	Met	M
Aspartic Acid	Asp	D	Phenylalanine	Phe	F
Cysteine	Cys	C	Proline	Pro	P
Glutamine	Gln	Q	Serine	Ser	S
Glutamic Acid	Glu	E	Threonine	Thr	T
Glycine	Gly	G	Tryptophan	Trp	W
Histidine	His	H	Tyrosine	Tyr	Y
Isoleucine	lle	L	Valine	Val	V

Appendix 9: Result of individual sera reaction with synthesis peptides.

Black columns sera before infection & white columns sera after infection.

peptide 1
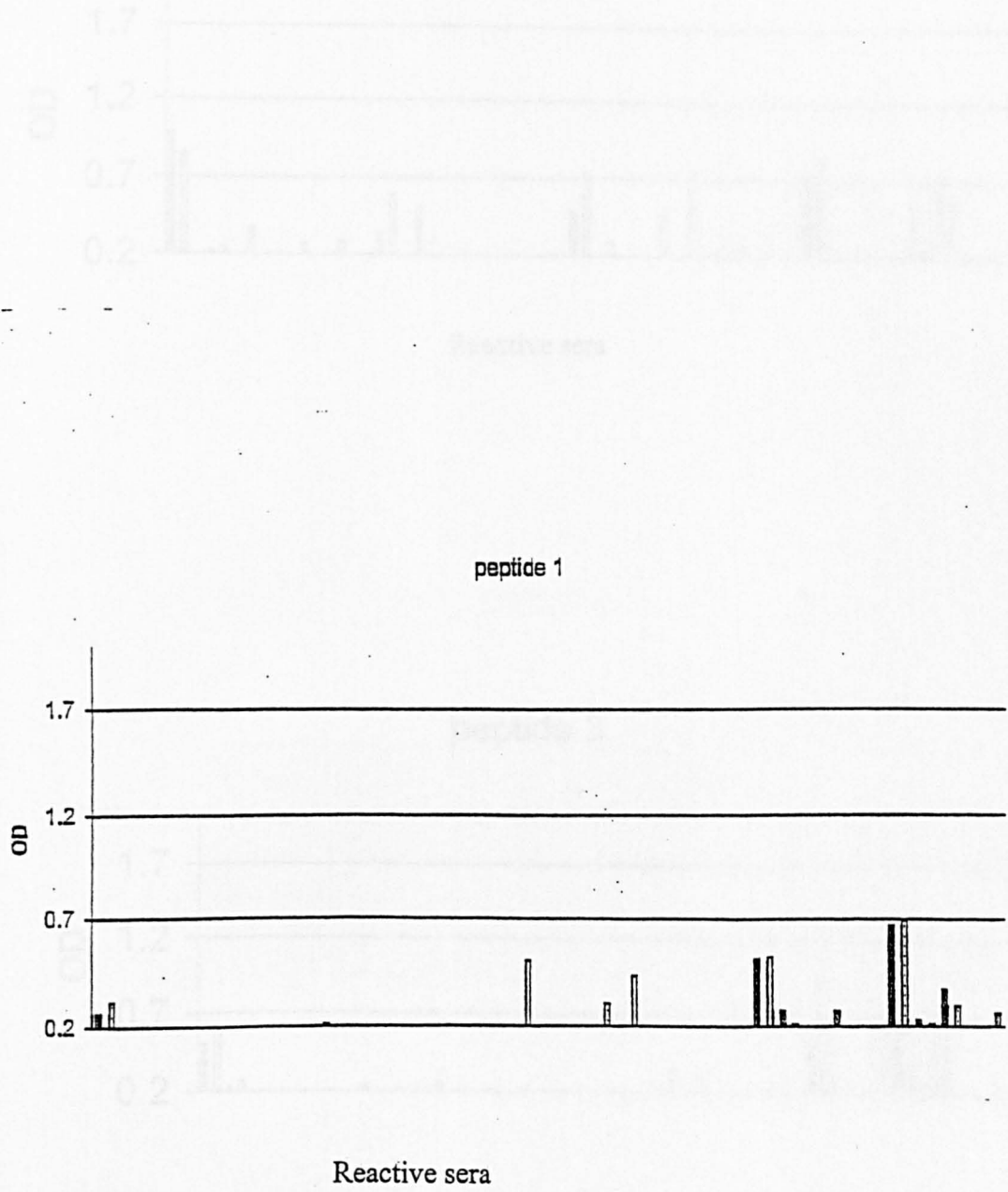
Reactive sera
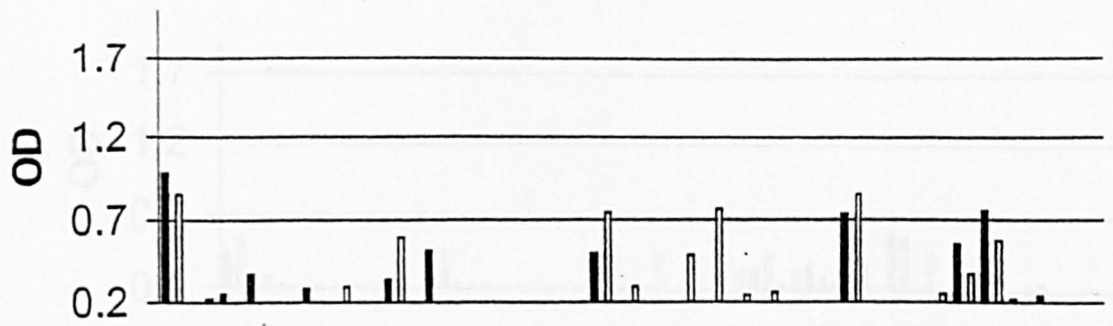
peptide 2

Reactive sera

peptide 3
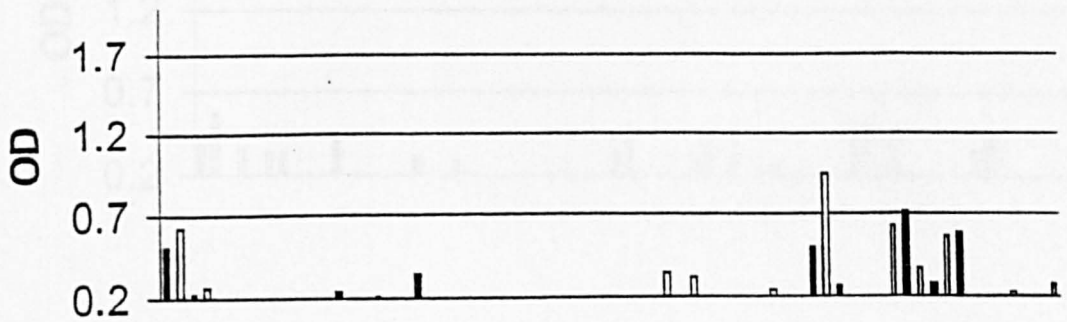
Reactive sera

peptide 4
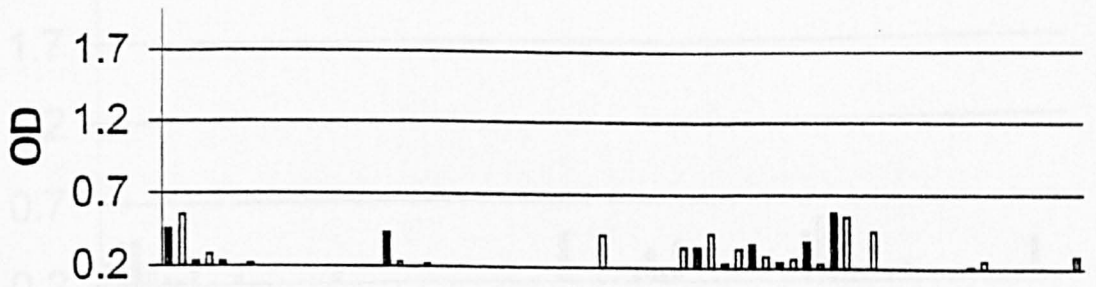
Reactive sera

peptide 5
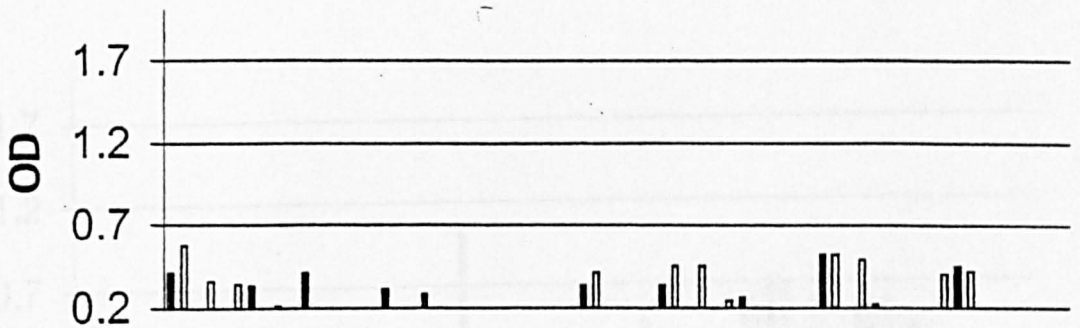
Reactive sera

peptide 6
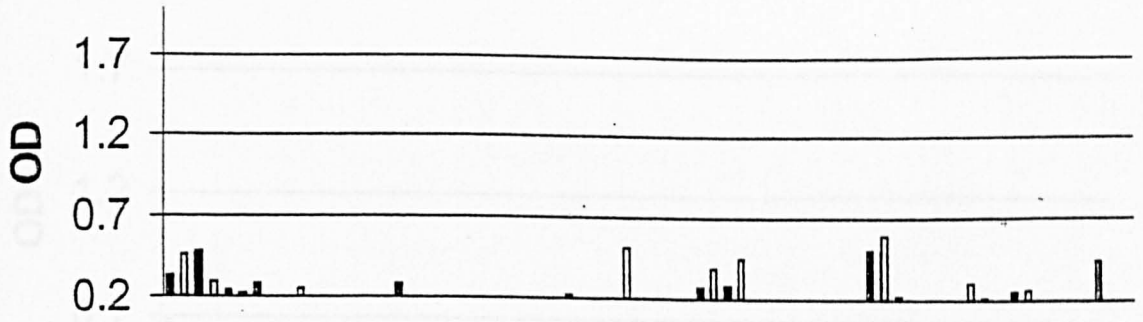
Reactive sera

peptide 7
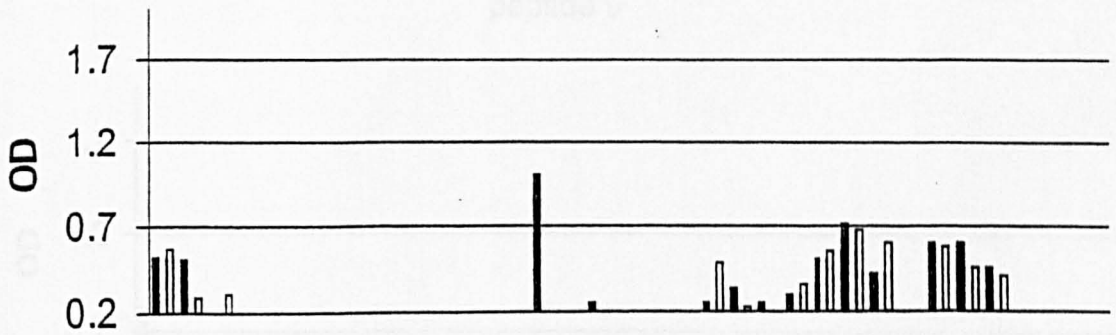
Reactive sera

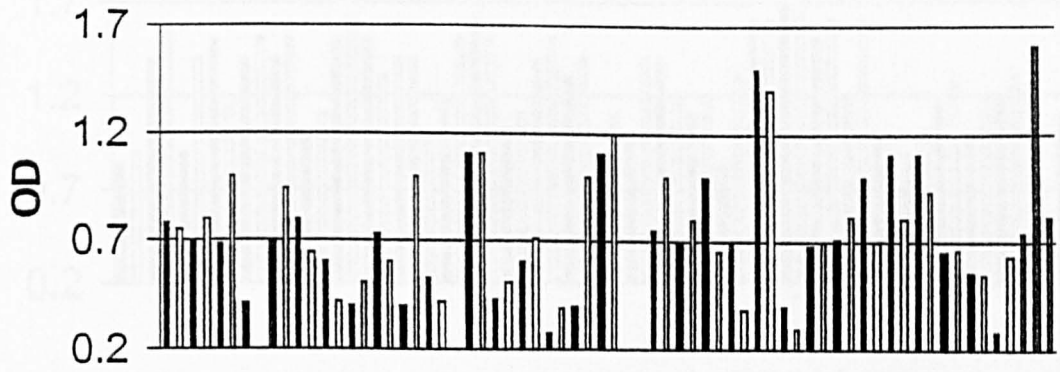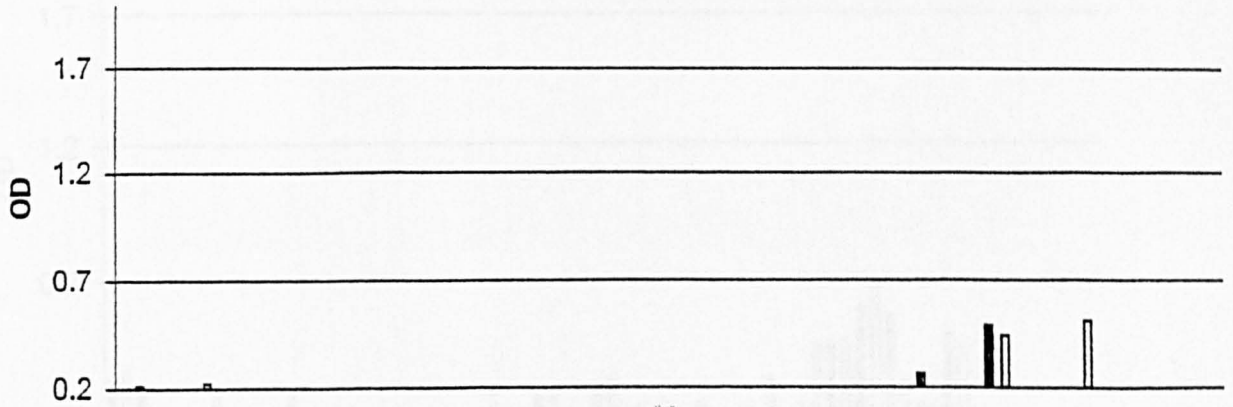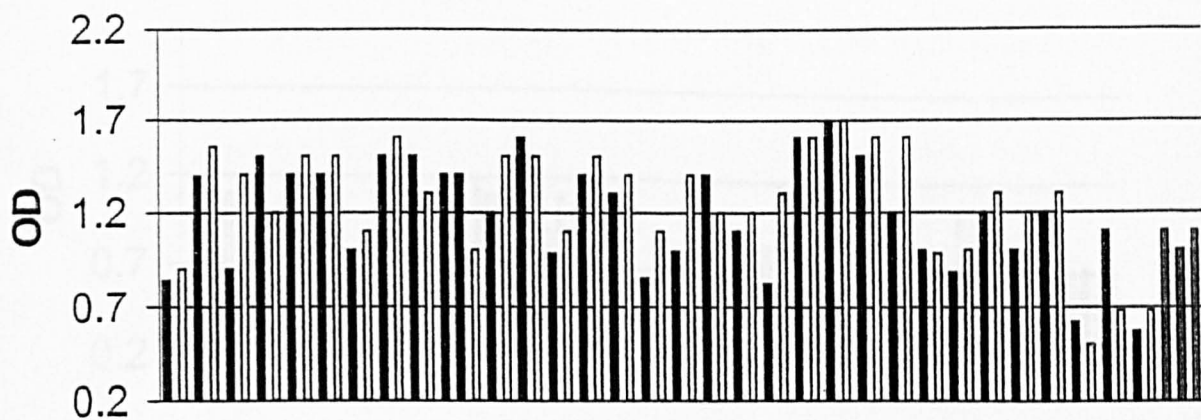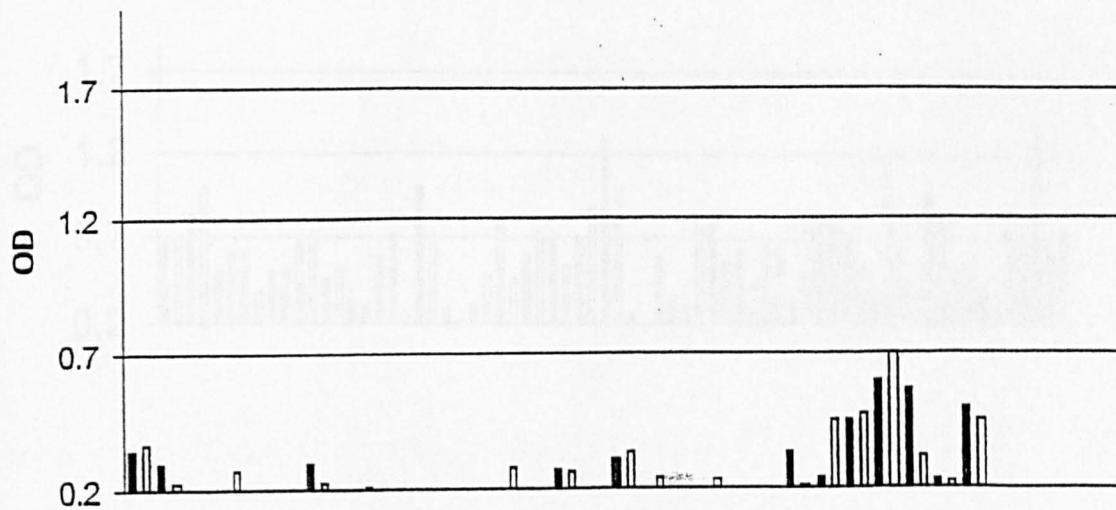# peptide 8
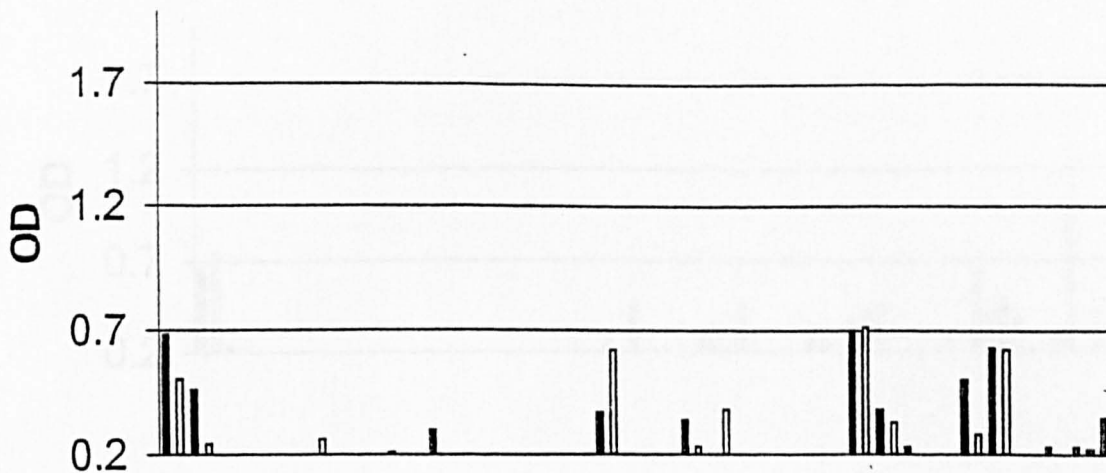
Reactive sera

peptide 9
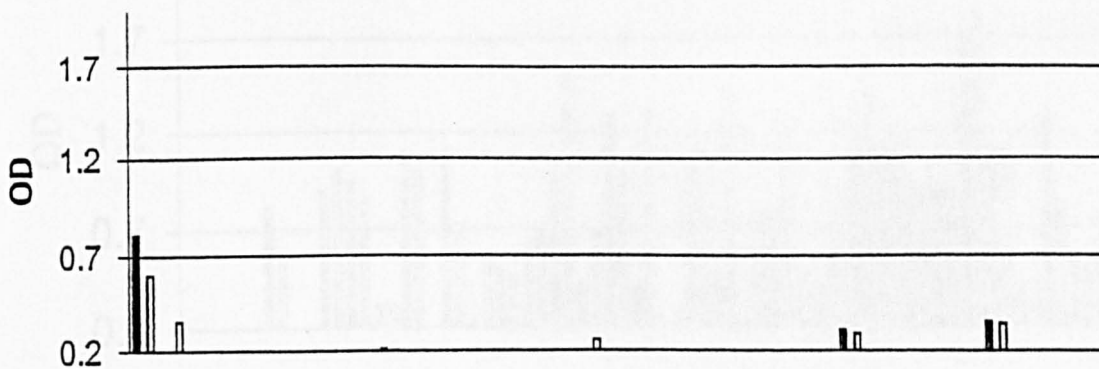
Reactive sera

peptide 10
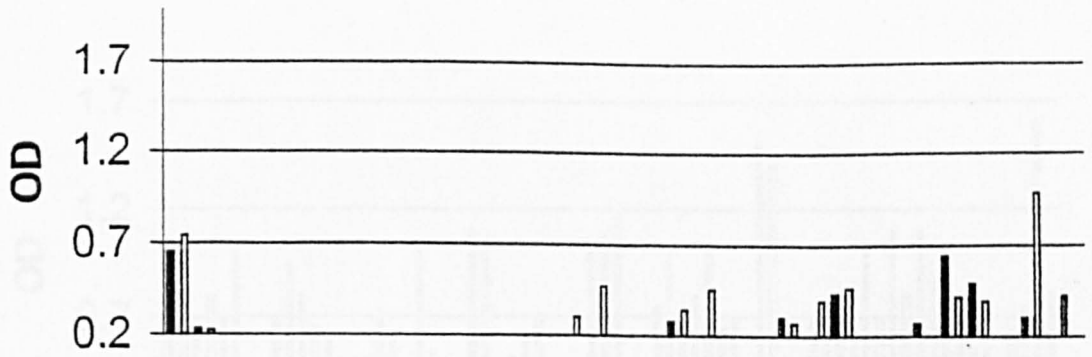
Reactive sera

peptide 11
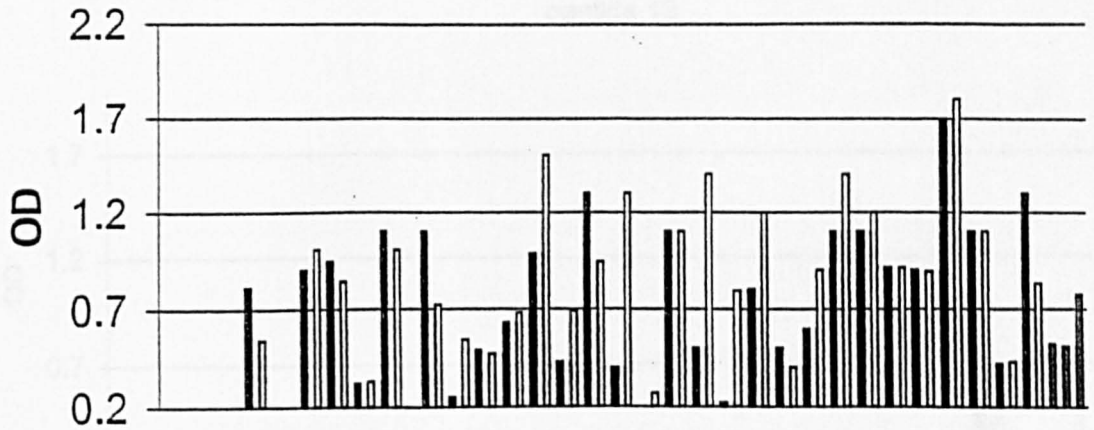
Reactive sera

peptide 12

Reactive sera

peptide 13

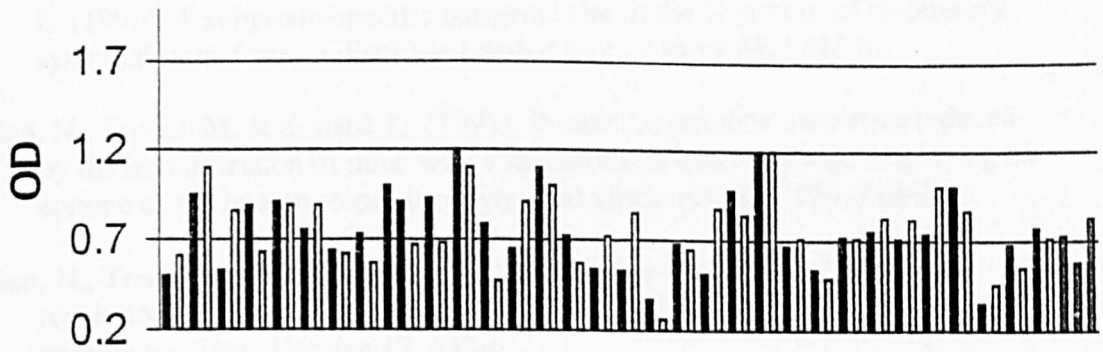Reactive sera
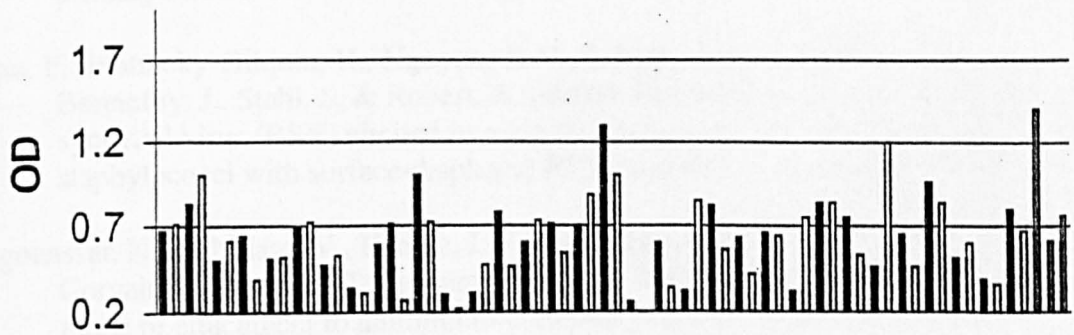
peptide 14

Reactive sera

peptide 15

Reactive sera

peptide 16

Reactive sera

peptide 17

Reactive sera

Appendices references

Akerlind-Stopner, B., Utter, G., Mufson, M. A., Orvell, C., Lerner, R. A. & Norrby, E. (1990). A subgroup-specific antigenic site in the G protein of respiratory syncytial virus forms a disulfide-bonded loop. *J Virol* 64, 5143-8.

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