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Epidemiologic and genetic studies of paramyxoviruses

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CONTENTS

1	Summary	4
2	List of original papers.....	5
3	List of abbreviations.....	6
4	Background	8
4.1	History.....	8
4.2	Classification.....	9
4.3	Replication strategy.....	11
4.4	Pathogenesis.....	12
4.5	Immune response	14
4.6	Clinical aspects	16
4.7	Epidemiology	18
4.8	Diagnosis.....	20
4.9	Treatment and prevention	22
5	Aims of the study	24
6	Materials and Methods	25
6.1	Materials for analysis	25
6.2	Methods.....	27
6.2.1	RNA isolation	27
6.2.2	Reverse Transcriptes (RT)-PCR and sequence analysis	27
6.2.3	Indirect immunofluorescence microscopy.....	27
6.2.4	Probe design and melt curve analysis.....	28
7	Results and discussion.....	29
7.1	Study on mumps virus neurovirulence.....	29
7.2	Study on the molecular epidemiology of RSV in Stockholm	30
7.3	To develop a novel method based on RT-PCR followed by	32
	two-dimensional melting curve analysis based on FRET	
7.4	Study on the epidemiology of hMPV in Stockholm	34
8	Conclusions	37
9	Populärvetenskaplig sammanfattning.....	38
10	Acknowledgements	41
11	References	42
12	Appendix	58

1 Summary

Paramyxoviruses are viruses of the paramyxoviridae family of the mononegavirales order; they are negative-sense single-stranded RNA viruses responsible for a number of diseases in humans and animals.

Mumps virus belongs to the Rubula virus genus. Mumps is a common childhood infection. The central nervous system (CNS) is a common site of mumps virus dissemination with or without signs of parotid gland involvement. In recent years the degree of neuropathogenicity has been estimated for specific mumps virus strains. The symptoms of mumps have been compared with individual virus strains /genotypes in clinical settings, and there has been evidence that certain virus strains are more neurovirulent than others. Thirteen different genotypes of mumps virus small hydrophobic (SH) gene, designated A to M, have been found in the beginning of the year 2008.

Respiratory Syncytial virus (RSV) is a member of the subfamily pneumovirinae and genus pneumovirus. RSV is the most common viral pathogen for lower respiratory tract infection among infants and young children, and it is recognized as an important agent for respiratory disease in the elderly and in transplant patients.

Antigenic characterization of RSV strains with monoclonal antibodies has identified two distinct groups of the virus, RSV group A and B, which circulate worldwide. The existence of eight group A genotypes, named GA1 to GA7 and SAA1 have been reported.

Human metapneumovirus (hMPV) also belonging to the subfamily pneumovirinae and genus metapneumovirus has been identified in 2001 in Netherlands. It is the only member capable of infecting humans in the genus metapneumovirus. Genotyping studies have determined that hMPV can be classified into two main lineages and four sublineages, named A1, A2, B1, B2. The frequency of the hMPV detection in patients with respiratory tract infection ranged from 1, 5 to 41%.

The aims of this thesis was (i) to investigate the sequences of the HN and F protein genes for the neurovirulent C1 and non-neurovirulent C2 variants and compare the results with the HN and F proteins of known genotypes. As the Kilham strain is neurovirulent, but other genotype A viruses are not, it was considered of interest to see if a mutation in the HN protein could explain its neuropathogenic capacity. (ii) to study the molecular epidemiology of RSV of group A isolated in Stockholm over a longer time period. (iii) to develop a simple and highly efficient method for genotyping group A of RSV. (iv) to study the epidemiology of hMPV in Stockholm, Sweden, and compare the epidemiology of hMPV with five other common respiratory viruses. During an epidemic in Lithuania in 1998-2000 mumps virus strains isolated were studied. Viruses of the C1 and C2 small hydrophobic (SH) genotypes variant were sequenced for the HN and F protein genes. Amino acid differences between C1 and C2 strains were found for both proteins. Two amino acid differences were of potential importance for the non-neurovirulent phenotype of the C2 virus. These amino acid differences have previously been reported to associate with a change in neurovirulence and fusion activity. In addition, the HN gene of the neurovirulent Kilham strain of genotype A was sequenced. The deduced amino acid sequence showed different amino acids compared to both genotype A and genotype C on some positions.

The epidemiology of respiratory syncytial virus (RSV) group A was followed by nucleotide sequencing of the variable parts of the glycoprotein (G) gene. The amino acid sequences of an amino-terminal and carboxy-terminal amino acid portion of the G protein in 47 virus strains collected in Stockholm, between 1965 and 2004, were determined. Phylogenetic analysis jointly with previously described genotypes (GA1 to GA7 and SAA1), showed that 34 virus strains belonged to genotype GA5, seven to GA2, three to genotype GA1, one to genotype GA4 and two to genotype GA7. Genotype GA5 was predominant in four epidemics, between 2000/2001 and 2003/2004.

Genotyping of respiratory syncytial (RS) virus group A by the use of a novel method based on reverse-transcriptase polymerase chain reaction (RT-PCR), FRET (fluorescence resonance energy transmission) based detection and two-dimensional melting curve analysis was applied on eighty RS virus samples of group A collected in Stockholm from 1976 to 2005. The T_m values were assessed for three different genotypes (GA2, GA5 and GA7) circulating in Sweden using two pairs of probes and subsequent data analysis by plotting results in a two-dimensional system. The results obtained were compared to genotyping by conventional nucleotide sequencing and phylogenetic tree analysis. It was found that the new assay was able to make a correct genotype identification in about 89% of the isolates and identified the remaining 11% as untypeable and candidates for conventional nucleotide sequencing.

The human metapneumovirus (hMPV) was analyzed retrospectively, by RT-PCR in five epidemic seasons, 2002-2006. After respiratory syncytial virus (RSV), influenza A virus and parainfluenza virus hMPV was the fourth most common respiratory virus with a detection rate of 2,9% (n=143/4,989) in nasopharyngeal samples. genotype A dominated over genotype B.

Approximately 2,6% (n=68/2,579) of the hMPV positive patients were <3 years, 3,1% (75/2,410) were ≥3 years and 2,5% (n=52/2,122) were <1 year. This age distribution differed from RSV, influenza A, B and parainfluenza virus. hMPV epidemics peaked in March, not coincident with RSV or parainfluenza virus. Large hMPV virus epidemics occurred biannually and were anticyclical to RSV epidemics.

2 LIST OF ORIGINAL PAPERS

This thesis is based on the following original papers, which will be referred to in the text by their roman numerals:

I: **Farideh Rafiefard**, Bo Johansson, Tesfaldet Tecle and Claes Örvell. Characterization of mumps virus strains with varying neurovirulence. *Scandinavian Journal of Infectious Diseases* 2005; 37:330-337.

II: **Farideh Rafiefard**, Bo Johansson, Tesfaldet Tecle and Claes Örvell. Molecular epidemiology of respiratory syncytial virus (RSV) of group A in Stockholm, Sweden, between 1965 and 2003. *Virus Research* 2004; 105:137-145.

III: **Farideh Rafiefard**, Claes Örvell and Kåre Bondeson. Genotyping of respiratory syncytial virus (RSV) group A in Stockholm, Sweden, using PCR and two-dimensional melting curve analysis. *Acta Pathologica, Microbiologica et Immunologica Scandinavica* 2008; 116:317-322.

IV: **Farideh Rafiefard**, ZhiBing Yun and Claes Örvell. Epidemiologic characteristics and seasonal distribution of human metapneumovirus infections in five epidemic seasons in Stockholm, Sweden, 2002-2006. In press, *Journal of Medical Virology*. 2008.

3 LIST OF ABBREVIATIONS

ABI	Applied Bio System
A-terminal	amino-terminal
bp	base pair
C-DNA	Complementary- deoxyribonucleic acid
CF	complement fixing
CNS	central nervous system
CSF	cerebrospinal fluid
C-terminal	carboxy-terminal
CTL	cytotoxic T lymphocyte
DNA	deoxyribonucleic acid
ELISA	enzyme linked immunosorbent assay
F	fusion
FA-buffer	fluorescence buffer
FRET	fluorescence resonance energy transmission
G	glycoprotein
GMK	Green Monkey Kidney
hMPV	human metapneumovirus
HN	hemagglutinin-neuraminidase
IF	immunofluorescence
IFN- α -2 β	interferon-alpha-2 beta
IgA	immunglobulin A
IgE	immunglobulin E
IgG	immunglobulin G
IgM	immunglobulin M
ILI	influenza like illness
L	large
Lo	London
LRTI	lower respiratory tract infection
M	membrane
M2.1	transcription elongation factor
M2.2	RNA synthesis regulatory factor
Mab	monoclonal antibody
MDCK	Madin-Darby Canine Kidney
MEM	minimal essential medium
mRNA	messenger ribonucleic acid
N	nucleoprotein
NP	Nucleocapsid protein
N-terminal	amino-terminal
P	phosphoprotein
PIV	parainfluenza
PBS	phosphate buffered saline
PCR	Polymerase chain reaction
RNA	ribonucleic acid
RSV	respiratory syncytial virus
RT	reverse trascriptase
RTI	respiratory tract infection
SARS	Severe Acute Respiratory Syndrome
SH	small hydrophobic

SIIDC	Swedish Institute for Infectious Disease and Control
T _m	melting temperature
tMK	tertiary monkey kidney
Y	year

Amino acid letter symbols

Amino acid	three letter code	one letter code
Alanine	Ala	A
Cysteine	Cys	C
Aspartic acid	Asp	D
Glutamic acid	Glu	E
Phenylalanine	Phe	F
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Lysine	Lys	K
Leucine	Leu	L
Methionine	Met	M
Asparagine	Asn	N
Proline	Pro	P
Glutamine	Gln	Q
Arginine	Arg	R
Serine	Ser	S
Threonine	Thr	T
Valine	Val	V
Tryptophan	Trp	W
Tyrosine	Tyr	Y

4 BACKGROUND

4.1 History

Mumps- In the 5th century B.C., Hippocrates described a mild epidemic illness associated with nonsuppurative swelling near the ears and, variably, with painful swelling of one or both testes. The name mumps may derives from an old English verb that means to grimace, grin or mumble [1, 2]. Hamilton a physician of the late 18th century, is credited as being the first to associate central nervous system (CNS) involvement with mumps in his description of the neuropathology of a fatal case [3]. Johnson and Goodpasture [4] convincingly showed that mumps was the result of a viral infection. They reported that filtered parotid secretions from four of six patients with mumps could induce parotid swelling when injected into the orifices of the ducts of Stensen of rhesus monkeys. The demonstration that mumps virus could be isolated in, as well as propagated to adequate titer in embryonated eggs [5] resulted in resolution of a number of physical properties of the virus. Demonstration of efficient biological activities of the virus such as hemagglutinating [6], haemolytic [7], and neuraminidase [6], activity and tissue culture propagation [8], allowed a more detailed understanding of mumps virus and the epidemiology and pathogenesis of mumps. An attenuated mumps virus vaccine was introduced in the United States in 1967 [9]. This resulted in a steady decline in the incidence of reported mumps cases, from 76 per 100,000 population in 1968 to the low level of less than one reported case per 100,000 population [10, 11]. However, the use of mumps vaccine still remains relatively restricted to more highly developed countries. Therefore, mumps is likely to remain a common worldwide human viral infection for some time.

Respiratory syncytial virus- RSV was first isolated in 1956 from a symptomatic laboratory chimpanzee during an outbreak of illness resembling the common cold [12]. Shortly thereafter, the same virus was recovered from infants with respiratory illness, and serologic studies indicated that infection in infants and children was common [13]. RSV quickly became recognized as the most important viral agent of serious respiratory tract disease in the pediatric population worldwide [14-20]. RSV is receiving increasing recognition as an important agent of disease in the elderly and in bone marrow transplant recipients [21-24] as well as in the general adult population [25]. Pediatric RSV disease claims about a million lives annually, and no reliable antiviral or vaccine currently exist [26, 27]. RSV vaccine developed in the 1960s proved to be disappointingly non-protective and actually led to more

severe lung disease in vaccinated children who subsequently acquired natural RSV infection. Children who received the vaccine developed high levels of virus-specific antibodies, which however, were of low neutralizing titer [28] and failed to protect against infection by RSV [29-32].

Human metapneumovirus-hMPV is one of the most recently identified human respiratory viral pathogens [33] in individuals of all ages [34] but it is especially prevalent in children [35]. In 2001, van den Hoogen et al. reported the isolation of a novel paramyxovirus, human metapneumovirus (hMPV) from children with respiratory tract disease [33]. hMPV has been found worldwide [34, 36-39] and appears to have a seasonal distribution (winter and spring) [40].

There is still no reliable antiviral or vaccine available against hMPV.

4.2 Classification

The family Paramyxoviridae has been reclassified in 2000 by the International Committee on the Taxonomy of viruses into two subfamilies, the Paramyxovirinae and the Pneumovirinae.

Paramyxovirinae contains three genera: Respirivirus, Rubulavirus and Morbillivirus.

Pneumovirinae contains the genera: Pneumovirus and metapneumovirus (Figure 1) [41].

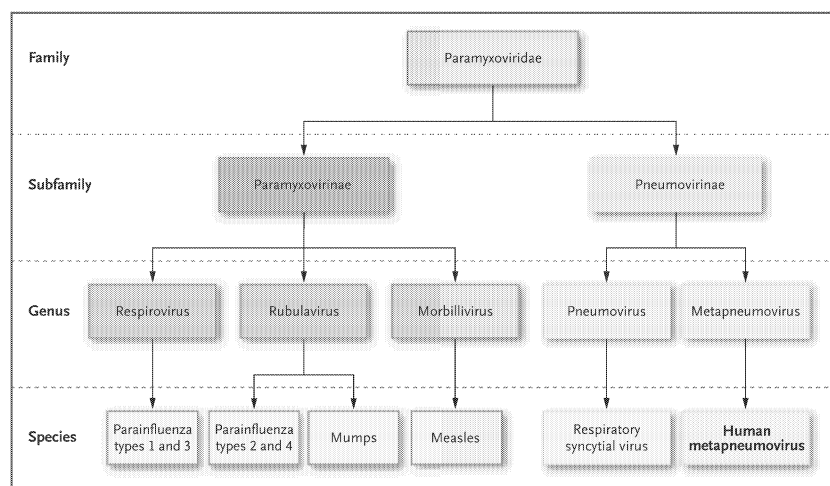


Figure 1. Classification of viral pathogens of the paramyxoviridae family that infect humans

The new classification is based on morphologic criteria, the organization of the genome, the biologic activities of the proteins, and the sequence relationship of the encoded proteins. The pneumoviruses can be distinguished from Paramyxovirinae morphologically as they contain nucleocapsids with a smaller diameter. In addition, the number of encoded proteins differs and the pneumoviruses also contain an attachment protein that is very different from that of Paramyxovirinae [42].

Paramyxoviruses contain nonsegmented, single strand RNA genomes of negative polarity, and they replicate entirely in the cytoplasm. Their genomes are 15 to 19 kB in length, and the genomes contain six to ten tandemly linked genes (Figure 2) [42].

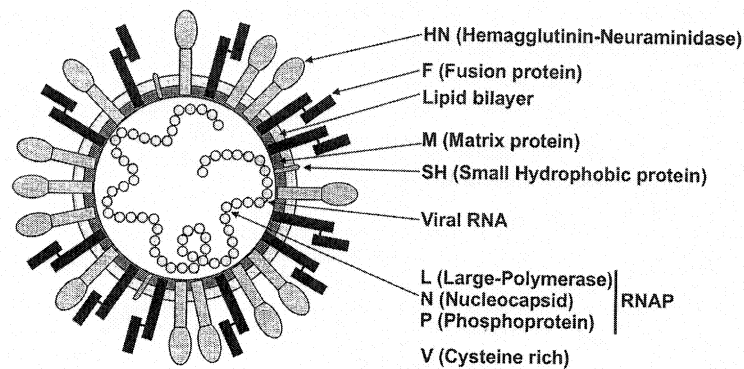


Figure 2. Schematic diagram of a member of the paramyxoviridae family

Mumps virus belongs to the Rubulavirus genus in the family Paramyxoviridae [43, 44]. It has a single-stranded, negative-sense RNA genome which is 15,384 nucleotides long and contains seven genes that code for nine proteins: nucleocapsid (NP), phosphoprotein (P), V, I, matrix (M), fusion (F), small hydrophobic (SH), hemagglutinin-neuraminidase (HN) and RNA polymerase (L) [45]. All mumps virus genes have been sequenced [46-51]. The small hydrophobic (SH) gene of mumps virus was identified as a membrane protein in 1996. It has not yet been identified as a structural protein in extracellular virions [52]. Phylogenetic comparison of the nucleotide sequences of the SH genes from different mumps virus isolates from around the world have shown the existence of thirteen genotypes, which have been named A-M. [53-55].

Respiratory syncytial virus (RSV) belongs to the Pneumovirus genus in the family Pneumovirinae. The complete genome is approximately 16,000 nucleotides in length and encodes eleven genes in the following order on the genome map: putative non-structural protein (NS1)- putative non-structural protein (NS2)- nucleocapsid (N)- phosphoprotein (P)- matrix protein (M)- small hydrophobic transmembrane (SH)- attachment glycoprotein (G)- fusion glycoprotein (F)- transcription antitermination factor (M2-1)- RNA regulatory protein (M2-2)- RNA polymerase (L) [56]. Antigenic characterization of RSV strains with monoclonal antibodies has identified two distinct groups of the virus, RSV group A and, B, which circulate worldwide [57, 58].

Respiratory syncytial virus A has been reported to contain eight different genotypes, GA1 to GA7 and SAA1 on the basis of classification of the C-terminal variable region of the G protein [59-62].

Human metapneumovirus (hMPV) belongs to the pneumovirus genus in the family pneumovirinae [33]. hMPV consists of 13,350 nucleotides and nine proteins: the nucleoprotein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), transcription antitermination factor (M2-1), RNA synthesis regulatory factor (M2-2), small hydrophobic protein (SH), attachment protein (G) and major polymerase subunit (L) in the order 3'-N-P-M-F-M2.1-M2.2-SH-G-L-5' [63].

Two major groups A and B have been initially identified based on distinct reactivity with monoclonal antibodies and four subgroups, named A1, A2, B1 and B2 [64].

4.3 Replication strategy

All aspects of the replication of Paramyxoviridae take place in the cytoplasm. Their genomes are 15 to 19 kB in length, and the genomes contain six to ten tandemly linked genes. A lipid envelope containing two surface glycoproteins (F and HN, or H or G), which mediate the entry and exit of the virus from its host cell, surrounds the virions. Inside the envelope lies a helical nucleocapsid core containing the RNA genome and the nucleocapsid (N), phospho- (P), and large (L) proteins, which initiate intracellular virus replication. Residing between the envelope and the core lies the viral matrix (M) protein which is important in virion architecture and that is released from the core during virus entry.

Intracellular replication of paramyxoviruses begins with the viral RNA-dependent RNA-polymerase (minimally, a homo tetramer of P and a single L protein molecule) transcribing

the N-encapsidated genome RNA into 5' capped and 3' polyadenylated mRNAs. The viral RNA-polymerase begins all RNA synthesis at the 3' end of the genome, and it transcribes the genes into mRNAs in a sequential manner by terminating and reinitiating at each of the gene junctions. The junctions consist of a gene-end sequence, at which polyadenylation occurs by the reiterative copying of four to seven uridylates, a short non transcribed intergenic region, and a gene-start sequence that specifies capping as well as mRNA initiation. The polymerase occasionally fails to reinitiate the downstream mRNA at each junction, leading to the loss of transcription of further-downstream genes, and hence there is a gradient of mRNA synthesis that is inversely proportional to the distance of the gene from the 3' end of the genome. After primary transcription and translation, when sufficient amounts of unassembled N protein are present, viral RNA synthesis becomes coupled to the concomitant encapsidation of the nascent (+) RNA chain. Under these conditions, the polymerase ignores all the junctions, to produce an exact complementary antigenome chain, in a fully assembled nucleocapsid (Figure 3) [65].

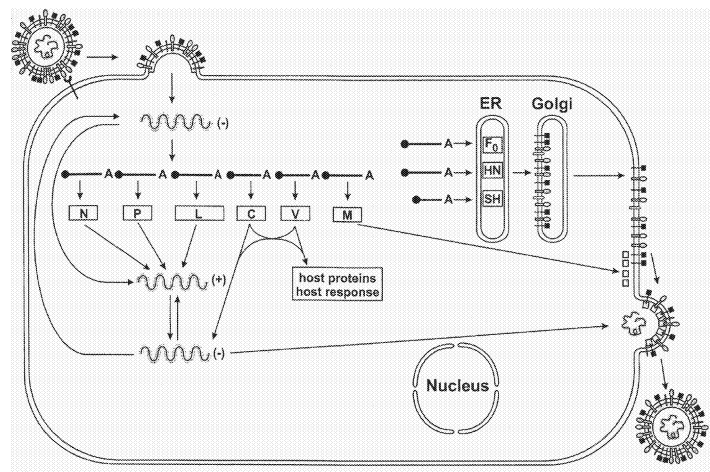


Figure 3. Schematic representation of the life cycle of a paramyxovirus

4.4 Pathogenesis

Mumps- Susceptible individuals in close but not necessarily intimate contact are required to establish an epidemic [66]. Transmissibility after nasal or buccal mucosal inoculation of virus suggests that natural infection is initiated by droplet spread [4]. During the 18 days incubation period the virus multiplies in the upper respiratory mucosa, spreads to draining lymph nodes,

and then disseminates via a transient plasma viremia [67, 68]. Virus is shed in saliva for as long as 6 days before the onset of parotitis. The viral excretion stops when local secretory IgA antibodies appear, which happens as early as 5 days after the start of symptom [69]. Virus-specific IgM antibodies are also present early in saliva [70] and patients with mumps are infectious for a time period of totally about 7 to 10 days. Plasma viremia appears to be terminated by the developing humoral antibodies response [67], which can be detected in serum as early as 11 days following experimental infection of humans. Mumps virus preferentially infects activated human T lymphocytes [71]. Animal models suggest that circulating infected lymphocytes provide a means for the spread of virus in the face of mounting humoral immunity [72].

Respiratory syncytial virus- The incubation period from time of infection to onset of illness for RSV is 4 to 5 days [73, 74]. It is generally thought that there is a positive correlation between the level of virus replication and the disease.

RSV replicates primarily in the superficial layer of the respiratory epithelium [75, 76]. It also infects macrophages and monocytes and appears to interfere with some of their functions [77]. Viral spread from the upper to the lower respiratory tract likely involves aspiration of secretions or spread via the respiratory epithelium. The virus also can spread from cell to cell by fusion without emergence into the extracellular fluid, although the importance of this mechanism is unclear in vivo. Lower respiratory tract signs usually appear 1 to 3 days after the onset of rhinorrhea, and it is assumed that this reflects the timing of viral spread into the bronchi and bronchioles. The titer of virus in the lower respiratory tract is not known, but based on studies in chimpanzees it likely is similar to that in the upper respiratory tract. IgE is involved in the pathogenesis of RSV disease. In some studies, the level of free IgE in secretions from infected persons during convalescence from RSV infection was higher in individuals with asthma and wheezing and was correlated with the severity of hypoxia during acute disease [78, 79]. Moreover, increased levels of histamine and leukotrienes were detected in secretions of patients with bronchiolitis or RSV-induced asthma but not in those of patients with either RSV pneumonia or upper respiratory illness [80]. These soluble factors are mediators of bronchoconstriction and presumably were released during the interaction of IgE, RSV, and mast cells in the respiratory epithelium. However, other studies did not find convincing evidence of increased IgE expression or mast cell degranulation [81, 82], and thus a general role for IgE in RSV pathogenesis remains uncertain.

Human metapneumovirus- The virus is thought to replicate in both the upper and lower respiratory tract, but the particular cell types that are affected have not been defined [83]. Several studies have quantified secreted host factors in the respiratory secretions of infected persons, such as the chemokine interleukin-8, [84, 85] and hMPV appears to elicit lower mucosal levels of inflammatory cytokines than does RSV [83]. The interpretation of these studies is not clear at this time. Recent studies in infected macaques suggest that ciliated epithelial cells are most affected, with antigen concentrated at the apical surface of polarized epithelial cells. However, antigen could be detected throughout the respiratory tract [86]. Naturally acquired infection does not appear to protect against re-infection. Symptomatic lower respiratory tract infection has been documented in sequential years in children [87]. Virtually every child exhibits serologic evidence of infection by age 5 years [33]; however, infections affecting adult persons are common. Nevertheless, it is likely that infection does induce partial protection against severe disease, because most serious lower respiratory tract illnesses occur in the first year of life [87].

4.5 Immune response

Mumps- The host's immune response to mumps virus infection is complex but the appearance of specific IgM, IgA and IgG antibodies follows the sequence usual for many acute viral infections. Both humoral and cell mediated immunity is probably important in the recovery process. In vitro lymphocyte-proliferative responses to mumps virus antigens are readily measured in seropositive individuals [88]. Cell-mediated responses to previously encountered antigens may be reduced during and for up to four weeks following human infection with attenuated mumps vaccine virus and wild mumps virus [89]. Following infection with wild-type or vaccine mumps virus strains [90, 91] CD8⁺ and gamma-delta cytotoxic T lymphocytes have been demonstrated in blood and/or CSF. The cellular response is important in the recovery from most human viral diseases, but with mumps there is little evidence to support that the development of cellular immunity is significant for recovery. However, some contribution of altered immune surveillance on the course of mumps has been suggested, for example by a report of persistence of mumps virus antigen in lymph nodes and the spleens of patients with Hodgkin's disease [92, 93]. Neutralising antibodies administered several days after a primary virus inoculation can save the animals from a lethal mumps virus infection [94].

Respiratory syncytial virus- The adaptive immune system has the primary role in recovery from RSV infection and resistance to re-infection [95].

In infants, where infection is more severe and longer in duration, the decrease in virus shedding in nasal secretions was associated with the appearance of RSV-specific IgA antibodies [96]. RSV-specific cytotoxic cells, thought to be CD8⁺ T lymphocytes, have been detected in peripheral blood mononuclear cells from infants with RSV disease [97]. It is reasonable to assume that, as in experimental animals, they are important in resolving RSV infection in humans. Thus both cellular and humoral immunity contribute to resolving RSV infection.

With regard to resistance to re-infection, immunization of BALB/C mice with recombinant vaccinia viruses that each expresses an individual RSV protein showed that F and G are the only proteins that induce RSV-neutralizing antibodies and long-lived resistance to challenge RSV replication [98]. Immunization with antigens that stimulated RSV-specific CD8⁺ CTLs but not neutralizing antibodies conferred resistance to subsequent infection, but this was short lived and had largely waned by 28 days [98 -100]. Thus antibodies mediate long-term resistance to re-infection, where memory CD8⁺ CTLs induced by primary infection did not augment resistance to re-infection, at least not in the BALB/c mouse. RSV infection does not induce significant levels of type I interferon in nasal secretions [101,102].

Complete cross immunity between the two groups of RSV has not been proved. One can speculate that both groups should be incorporated into any RSV vaccine to achieve the broadest possible representation of immunogens. Observations on subgroup-specific immunity are needed to assess the extent of cross immunity, if any, between the two subgroups and the importance of subgroup-specific immunity in modulating infection [103].

Human metapneumovirus- Very little is known about the mechanisms of immunity of hMPV infection, but it is likely that immunity will function in a manner similar to that against other Paramyxoviruses. Infection induces serum neutralizing antibodies in most experimentally infected animals that have been tested, and protection against re-infection has been induced by primary infection of hamsters. [104]. Although mice appear to exhibit relatively low permissivity for viral replication, they offer a tractable model for studying immune mechanisms, and experiments are going on in this area.

It is a well known fact that the greatest genetic diversity between the two genotypes of hMPV is located in the G protein. To address this issue, animal models have been used. Skiadopoulos et al. used two hMPV strains to represent the two genotypes of hMPV.

Infection with a virus of one hMPV genotype protected animals against infection with a virus of the heterologous genotype. Protection was defined as an inhibition of hMPV replication in nasal turbinates or lungs in infected animals that were previously infected with a strain of hMPV [104]. Reciprocal cross-neutralization assay with postinfection serum demonstrated that each strain induced a high level of neutralising antibody to homologous and heterologous strains. The data suggest that cross-protective immunity occurs after infection with a virus of one genotype and that the F protein is the major antigenic determinant on the virus. Therefore, the two genotypes may not represent distinct serotypes. However, van den Hoogen et al. demonstrated that serum obtained from an animal infected with a representative virus of one genotype could not neutralize a virus of the heterologous genotype in vitro, suggesting that the two genotypes were, in fact, distinct serotypes [105].

4.6 Clinical aspects

Mumps- The incubation period is about 18 days, but approximately one third of all mumps virus infections occur without recognized symptoms. Moderate fever is present at the onset of disease, with fever abatement a few days later. The feature most characteristic of mumps is salivary gland swelling (95% of all symptomatic cases), particularly the parotid glands (90% bilateral involvement). Enlargement of individual glands usually is maximal within 48 hours, at which time pain becomes most prominent, and may persist for a week or more [106]. Virus is present in the saliva for several days before the onset of clinical disease [107] and for up to 5 days later [69]. The virus can be detected in saliva for several weeks after the onset of mumps [108].

A variety of organs can be symptomatically involved during the course of mumps. These include the testes, CNS, epididymis, prostate, ovary, liver, pancreas, spleen, thymus, heart, mammary glands, lungs, bone marrow and joints [109,110]. Mumps orchitis is more frequently unilateral than bilateral and is more likely to occur after puberty [111]. Mumps meningitis usually develops about 5 days following the onset of parotitis and is almost always marked by fever; with vomiting, neck stiffness and headache [112]. Aseptic meningitis occurs in 10% of patients with mumps but as many as 50% show abnormalities in the CSF [113]. Before vaccination mumps was the most frequent causative agent of aseptic meningitis in many countries [111]. Virus can be isolated from CSF during the first 2 to 3 days after onset and later specific antibodies can be demonstrated in CSF.

Respiratory syncytial virus- In the normal infant who encounters RSV for the first time at age 6 weeks to 9 months, RSV infection usually causes upper respiratory symptoms. In 25 to 40% of such infections, however, the respiratory tract below the larynx also becomes involved [74,114]. Bronchiolitis and pneumonia are the primary manifestations of lower respiratory tract disease, and probably represent a continuum of increasing severity of disease.

Asymptomatic infection in this young age group is probably uncommon. Cough may appear simultaneously, but it occurs more often after an interval of 1 to 3 days. At that time there also may be sneezing and a low-grade fever. Soon after the cough has developed, the child may begin to wheeze; if the disease is mild, the symptoms may not progress beyond this stage. Examination usually shows moderate tachypnea, diffuse rhonchi, fine rales, and wheezes. There is profuse rhinorrhea, intermittent fever, and frequently otitis media [115]. In more severe cases, the coughing and wheezing progress, and the child becomes dyspnoic. Severe lower respiratory tract illness is most common in patients younger than 9 months.

Bronchiolitis may occur in older infants and children, but it is usually seen in children with recurrent wheezing who carry the diagnosis of asthma. One study implicated RSV infection as a risk factor in the development of asthma [116], but other studies suggest that viral infections are not a major factor in the development of allergic airway disease [117,118]. There is increasing support for the view that severe RSV infection with subsequent chronic or recurrent respiratory tract sequela can be due to preexisting deficiencies. Acute RSV infections are common in adults [119].

Human metapneumovirus-hMPV seems to be an important respiratory pathogen that causes both upper and lower respiratory tract infections in children [33,120], who unlike adults are rarely asymptomatic, although most reports are biased toward descriptions of the severe symptoms in hospitalised subjects. The fact that most severe cases are found in pediatric patients suggests that naturally acquired infection induces partial protection against the disease [121]. The degree of severity seems to be related not only to age, but also to the strain causing the infection with a possible link between the A2 strain, which is the most frequent, and severe disease [120].

Diagnosis of hMPV infection may be made on the basis of signs and symptoms, ranging from rhinopharyngitis to bronchitis and pneumonia, and some patients may be admitted to intensive care units [33,121]. In a population of children in an emergency department for acute respiratory infection, hMPV caused signs and symptoms that sometimes resembled those of RSV infection (bronchiolitis, asthma exacerbation and pneumonia) and sometimes those of the influenza (fever and upper respiratory tract infection) [122].

4.7 Epidemiology

Mumps- In an unvaccinated population, immunity to mumps is usually acquired between the ages of 5 and 14 years, with maximal acquisition of humoral antibody occurring between 4 and 7 years [123]. In temperate zones, a seasonal variation is evident, the highest incidence being around winter and spring months. No such seasonal variation exists in countries with tropical climate. The distribution of mumps virus genotypes varies extensively between different countries and even in different geographical locations within the same country [124-126]. Different mumps virus genotypes have been shown to co-circulate in the same country [124,126]. Thirteen different genotypes of the mumps virus small hydrophobic gene, designated A to M, were found in the year 2008 [55]. Mumps virus outbreaks have been reported to occur in populations with high vaccine coverage [127]. Genotypes that have been isolated in Europe in different time periods are A, C, D, E, G, H and J [54,124-126,128,129]. In Japan, the B, D, G, H and J genotypes have been found [126,129, 130]. A mumps outbreak in Japan with a high incidence of aseptic meningitis was found to have been caused by genotype I [132]. Genotypes A, C and D have been identified in North America [132]. The F and I genotypes have been isolated from China, Japan and Korea [127] and the M genotype was identified in Brazil [55].

Respiratory syncytial virus- RSV has a worldwide distribution. Wherever appropriate studies have been performed, RSV has been found to be the major pediatric viral respiratory tract pathogen. Serious lower respiratory tract disease occurs more commonly in males than in female subjects [20]. RSV has a clear seasonality in temperate zones of the world. In urban centers, epidemics occur yearly in the late fall, winter, or spring but not during the summer [18,133,134]. Each RSV epidemics lasts approximately 5 months, with 40% of infections occurring during the month in the temporal centre of the outbreak [135]. Most outbreaks peak in February or March. It is not known why the virus disappears in the summer, and from where it reemerges in the fall and winter. In tropical or subtropical areas, epidemics usually occur during the rainy season [136].

A number of studies indicate that subgroup A and B viruses can alternate in prevalence during successive years [137-139]. One interpretation is that re-infection with the heterologous subgroup is favored because it can partially evade previously induced immunity. Consistent with this view, a subgroup shift was not evident when the initial infection occurred in children

younger than 6 months, a time when immune response are reduced because of maternal antibodies and immunologic immaturity [139].

RSV is a major cause of nosocomial infection [140]. The rate of hospital-acquired infection for infants and children during an RSV season was reported to range from 26 to 47% in newborn units and from 20 to 40% for older children. Hospital staff appears to play a major role in nosocomial spread of RSV infection.

Human metapneumovirus- Since human metapneumovirus initial description in 2001, hMPV has been isolated from individuals of all ages with ARTI in Europe, America, Asia, Australia and South Africa [87,122,142]. The incidence of infection in different studies varies from 1,5 to 25%, thus indicating that hMPV is a ubiquitous virus with a worldwide distribution [87,122,142]. The few seroprevalence surveys, from the Netherlands, Japan and Israel, have demonstrated that virtually all children are infected by the age of 5-10 years, which indicates that hMPV infection is acquired early in life [33,120]. However, as the range of antibody titres measured by immunofluorescence assay was higher in individuals aged >2 years than in children aged 6-24 months, it is possible that there is a booster effect as a consequence of re-infection with the same or a closely related virus. In addition, studies of samples collected previously have shown that hMPV is not a new pathogen, with serological evidence of human infection dating from 1958 in the Netherlands, and virus isolation during the last 10-20 years in Europe and Canada [120]. These findings suggest that hMPV has been in circulation for a long period, but has been recognised only recently because of the development of new diagnostic and research methods.

hMPV has a seasonal distribution, the greatest number of hMPV infections are usually diagnosed at the end of winter or in early spring [87,122,141]. It has also been demonstrated that different hMPV genotypes may co-circulate in the population during a single year [141,105].

The similar seasonal distribution of several other respiratory virus infections may result in co-infection with hMPV and other respiratory viruses, but the role that hMPV plays as a co-pathogen is still not understood completely.

4.8 Diagnosis

Mumps- The clinical diagnosis of mumps is seldom problematic in the presence of parotitis, even when the disease is not epidemic [142]. In the absence of parotitis or when salivary gland enlargement is rather inconspicuous with symptom of other visceral organ or CNS involvement predominating, laboratory confirmation of the diagnosis is required, even during an epidemic. Serological studies have remained the mainstay of diagnosis for a long time. Comparison of titres performed on the same day from serum taken at onset of clinical disease and convalescent serum samples obtained 2 to 4 weeks later usually shows the anticipated rise when hamagglutination-inhibition or neutralisation assays are used [143]. New research has demonstrated that it is difficult to diagnose mumps virus re-infection and also in the case of a prior vaccination with a mumps virus vaccine (144, 145).

ELISA is a sensitive specific, rapid and cost-effective test. Determination of virus-specific IgM and IgG levels on a single acute-phase serum provides an alternative when paired sera are not available [146]. A purified mumps virus strain was used as the viral antigen to evaluate specific total IgG [147]. Virus isolation is also used. Immunofluorescence technique confirms and specifies the presence of the mumps virus isolate in the culture [148].

Amplification and sequencing of gene segments from cell extracts of primary isolates can provide useful epidemiological information [149]. In many instances of mumps meningitis, PCR is the method of choice. The diagnosis can be confirmed on the basis of elevated CSF/serum antibody ratio from a sample set taken during illness, since mumps antibodies are uncommon in the CSF during other CNS infection [150].

Multiplex real-time RT-PCR assay for rapid mumps diagnosis has been used in a clinical setting. This assay used oligonucleotide primers and a Taq-Man probe targeting the mumps SH gene, as well as primers and a probe that targeted the human RNase P gene to assess the presence of PCR inhibitors and as a measure of specimen quality [151].

Respiratory syncytial virus- The development of RSV cytopathology requires 3 to 7 days or more, but detection can be made more rapid and efficient by immunofluorescence or immunostaining and the use of shell viral cultures [152-154].

Rapid methods involve the detection of viral antigen or nucleic acid in secretions [154]. In the direct immunofluorescent assay (DFA), exfoliated cells are reacted with fluorescent RSV-specific antibody and examined by microscopy. In the enzyme immunoassay (EIA), the

sample containing respiratory secretions is incubated briefly with RSV-specific antibodies that are immobilized on plastic, and antigen thus captured is detected by reaction with a second, enzyme-linked RSV-specific antibody. More recently developed assays based on reverse transcription and polymerase chain reaction are commercially available. These assays can equal or exceed the sensitivity of cell-culture or antigen-based assays, and the inclusion of appropriate additional primers allows simultaneous assay for multiple pathogens [155]. Serologic methods are used less frequently. In older children and adults, an increase in complement-fixing (CF), neutralizing, or ELISA serum antibodies is a fairly sensitive index of re-infection with RSV. Asymptomatic infections are usually undetectable serologically. Serologic tests in infants are less sensitive, particularly in those younger than 4 months, with ELISA being the most sensitive assay [156]. Serologic assays have been improved using ELISA with purified viral proteins or synthetic peptides [157], permitting the sensitive identification of immunoglobulin isotype or subclasses of RSV-specific antibodies.

Human metapneumovirus- Most studies have only detected reliable cytopathic effects in tertiary monkey kidney (tMK) and LLC-MK2 cells [33,120]. In the absence of commercially available antibodies, the cytopathic effect of hMPV can be confirmed by using RT-PCR to test infected supernatants.

RT-PCR has become the method of choice for the diagnosis of acute hMPV infection, because of the unavailability of rapid antigen detection tests, and the slow and restrictive growth of the virus [33,120]. Most PCR protocols as published to date rely on amplification of the L (major polymerase subunit), N (nucleoprotein), or F (fusion protein) gene using primer sequences derived mainly from the prototype strain 001 from the Netherlands [158]. New rapid and sensitive hMPV assay, based on real time PCR, allow amplification and detection of hMPV in ≤ 2 h [158]. Serological tests only permit a retrospective diagnosis and, because infection is almost universal in childhood, seroconversion or a ≥ 4 -fold increase in antibody titer must be demonstrated to confirm recent infection [33,120,159,160]. A recent serological survey of hMPV was based on the use of a novel ELISA with hMPV-fusion protein antigen expressed in recombinant vesicular stomatitis virus [160]. Detection of hMPV antigens in nasopharyngeal secretions by an immunofluorescent antibody test has also provided interesting results [161,162]. Because hMPV replicates poorly in the conventional cell cultures used for the diagnosis of respiratory viruses, it is relatively difficult to isolate and has probably circulated unreported for some considerable time.

4.9 Treatment and prevention

Mumps- Treatment of mumps and its various complications is generally symptomatic. In a controlled study, adult males presenting with parotitis were alternatively given either an intramuscular injection of 20 ml of gammaglobulin prepared from human convalescent serum or simply confined to a hospital for routine, nonspecific symptomatic therapy [163]. In the group that had received specific treatment, four out of 51 patients developed orchitis, compared to 14 out of 51 who had received symptomatic treatment. No protective effect was seen with gammaglobulin obtained from a normal donor serum pool [163]. This study suggests that immunotherapy with high-titer polyvalent or monoclonal antibody preparations could be useful in selected cases but should be used very early in the course of the illness. Experimental treatment of orchitis with IFN- α -2 β has been reported [164]. Prevention though an effective vaccination program remains the only rational approach to the control of mumps.

Respiratory syncytial virus- Treatment of severe RSV disease of the lower respiratory tract requires considerable supportive care: mechanical removal of secretions, proper positioning of the infant, administration of humidified oxygen, and in the most severe cases, respiratory assistance [165-167]. The use of bronchodilators is controversial but may be beneficial in older infants if wheezing is an important symptom. Corticosteroids are commonly used as anti-inflammatory therapy, although there is a general lack of data demonstrating its efficacy. The nucleoside analogue ribavirin was approved in 1986 for treatment of RSV infection [168,169]. Ribavirin has antiviral activity in cell culture and in cotton rats, although its mode of action remains unclear. Conversely evidence is lacking that ribavirin decrease mortality, the duration of hospitalization, or the need for supportive therapies or that it improves long-term pulmonary function [167,170]. In some studies there were trends suggesting that therapy was beneficial, although not statistically significant.

The success of parenterally administered RSV-neutralizing antibodies for RSV immunoprophylaxis of high-risk infants and young children suggested that RSV-antibodies also might be effective as antiviral therapy against an established infection. The passive parenteral infusion of serum-derived RSV-neutralizing antibodies into cotton rats or primates at the peak of RSV infection strongly reduced virus replication without evidence of any immunopathologic effect [171]. Direct administration of RSV-neutralizing antibodies into the respiratory tract, rather than by parenteral inoculation, also was effective in reducing pulmonary virus replication, and a comparable therapeutic effect was achieved by using a

160-fold smaller quantity of antibodies [172]. Intravenous injection of RSV-neutralizing antibodies into previously healthy or high-risk infants and children hospitalized for RSV disease substantially reduced the level of virus shedding, to levels comparable to those achieved with ribavirin, but produced little or no improvement in clinical outcome [173,174]. Initial studies of the aerosol route of administration provided similar findings [175], but additional evaluation is needed. As indicated earlier also combination therapy with antibodies and ribavirin is being evaluated.

Human metapneumovirus- No treatment is registered currently and no specific prevention procedures are recommended for the management of hMPV infection. Ribavirin and a polyclonal intravenous immunoglobulin preparation have been found to have similar in-vitro antiviral activity against both hMPV and RSV [176], but clinical studies are required to confirm these observations. However, given the well-known limitations of these medications (i.e., severe adverse events, difficult administration and high costs), they should be used with caution and probably considered only for treating immunocompromised patients with severe hMPV disease, as in the case of RSV infection. Furthermore, high-titred intravenous immunoglobulin preparations active against hMPV could be used in patients with severe disease [177].

5 AIMS OF THE STUDY

- i) To investigate the sequences of the HN and F protein genes for the C1 and C2 variants of mumps virus and compare the results with the HN and F proteins of known genotypes. As the Kilham strain is neurovirulent, but other genotype A viruses are not, it was considered of interest to see if a mutation in the HN protein could explain its neuropathogenic capacity. At the same time an explanation for the type-specific behaviour of this virus strain in neutralization experiments might be exposed.
- ii) To study the molecular epidemiology of RSV of group A isolated in Stockholm over a longer time period. While most studies reported previously have analysed only C-terminal sequences of the G protein, it was decided to study and compare two different variable regions of the protein located in the N-terminal and C-terminal ends of the G protein.
- iii) To develop a simple and highly efficient method for genotyping group A of RS virus. The novel method was developed based on RT-PCR followed by two-dimensional melting curve analysis based on FRET (fluorescence resonance energy transfer) hybridisation probe technology.
- iv) To study the epidemiology of hMPV for consequential virus epidemic seasons over a longer time period, and to compare the epidemiology of hMPV to five other common respiratory viruses.

6 MATERIALS AND METHODS

6.1 Materials for analysis

Paper I

Serum and saliva samples were collected from patients with mumps attending the hospital of Kaunas in Lithuania. The samples belonged to genotype C and they formed two different clusters within the C genotype, named C1 and C2 (178). In addition, the Kilham strain of genotype A was used for sequencing of the HN gene. Virus strains were isolated from saliva samples after passage in Green Monkey Kidney (GMK) tissue culture cells maintained in Eagle's minimal essential medium (MEM) containing 2% foetal calf serum. It was only possible to isolate a small number of samples from the total material. Therefore, in some cases the original materials were used directly for nucleotide sequencing.

Paper II

Forty-seven RSV virus isolates of group A were obtained from patients with respiratory tract infection. Thirty-two isolates were collected in the 2000/2001–2003/2004 epidemic seasons. Eleven samples were collected in the time interval between 1990 to 2000, two were from 1982, one sample was collected in 1976 and one in 1965. The virus isolates were collected in the Stockholm area, at the Central Microbiological Laboratory in Stockholm before 1992 and at Huddinge University Hospital, thereafter. Virus isolation was carried out in Hela or GMK cells under standard conditions, and the isolates were stored at -70°C . The typing of virus strains in group A and B was performed with monoclonal antibodies specifically directed against one of the groups [58, 179].

Paper III

Eighty RSV isolates of group A belonging to genotypes GA2, GA5 and GA7 were obtained from patients with respiratory tract infection. Seventy-nine isolates were collected from 1991 onwards, one was from 1976. Virus isolation was carried out in Hela or GMK cells under standard conditions, and the isolates were stored at -70°C . The typing of virus strains in group A and B was performed with monoclonal antibodies specifically directed against one of the groups (58, 179).

Paper IV

Five epidemic seasons for respiratory virus infections extending from the years 2002 to 2006 were investigated. Samples were collected from November to the end of May with the exception of the 2001/2002 season when samples from November and December of 2001 were not accessible. The presence of hMPV was examined in 4,989 samples. Two thousand six hundred fifty- nine (2659) nasopharyngeal samples were obtained from patients with respiratory tract infection negative for other respiratory viruses. In addition 2,330 available nasopharyngeal samples from patients positive for other respiratory virus infections were studied. Of the 4,989 samples, 933 were from 2001/2002, 870 from 2002/2003, 1069 from 2003/2004, 1027 from 2004/2005, 1090 from 2005/2006.

Positive virus findings for other respiratory viruses were made in 2,344 samples by immunofluorescence (IF) of cells from nasopharyngeal samples and virus isolation from the same samples according to accredited methods at the Karolinska University Hospital (Huddinge). Less than 2% of the samples were examined by immunofluorescence analysis only without viral culture. The viruses which were identified were respiratory syncytial virus, parainfluenza virus type 1, 2 and 3, adenovirus and influenza virus type A and B. The majority of the parainfluenza virus samples were typed with monoclonal antibodies. Because of the fact that some parainfluenza virus samples were not typed the parainfluenza virus infections were presented as one group. The recently described human bocavirus was not diagnosed [180]. Virus isolations were performed either in MDCK cells with 1µgr trypsin /ml in the serum free medium when the diagnosis of influenza virus infection was asked for or in four cell lines, Hela, GMK, MDCK and Rd cells when a specific influenza virus diagnosis was not asked for. Therefore samples subjected to routine diagnosis were not examined for all of the viruses mentioned in the foregoing. In order to make a better comparison between the occurrence of different viruses the positive findings were expressed in relative frequencies for each specific virus (number of positive samples/number of examined samples). The total material of positive and negative virus samples was divided into two groups. One of the groups consisted of samples from patients <3 years of age (infants and younger children) and the other group contained samples from patients ≥ 3 years (older children and adults).

6.2 Methods

6.2.1 RNA isolation (paper I, II, III, IV)

Extraction of RNA from the culture grown isolates was performed with QIAamp RNA minikit (QIAGEN, Hilden, Germany) following the manufacturer's instructions.

6.2.2 Reverse Transcriptase (RT)-PCR and sequence analysis (paper I, II, III, IV)

The described PCR method in paper I was used to amplify F and HN genes of mumps virus a 584 bp fragment of the G gene for NH₂-terminal, and a 327 bp fragment for the COOH-terminal part of the variable region of the G gene of RS virus (paper II, III) and a 110 bp of the L gene of hMPV (paper IV). Amplified products were purified with QIAquick PCR purification kit (Qiagen, Hilden, Germany) and sequenced with the same primers as were used for amplification. Nucleotide sequencing was carried out using the ABI analysis for genotype determination on the HN gene (paper I), a 168 nucleotides long stretch of the C-terminal region of the G gene (nucleotides from 753 to 921) (paper II, III), and a 110 nucleotides long stretch of the L gene (paper IV) using the TREECON program [181]. Sequence distances were calculated from alignments using the Jukes and Cantor option, and clustering was performed by the Neighbor-joining method of this program package. Evaluation of the robustness of the tree was performed by bootstrap analysis (100 trees).

6.2.3 Indirect immunofluorescence microscopy (paper II)

The presence of RS virus group A or B antigens in nasopharyngeal samples were determined by the direct immunofluorescence technique. Six monoclonal antibodies were used on cells smears to determine RS virus group identity.

The monoclonal antibodies (Mabs) used included two antibodies that reacted with the fusion glycoprotein F and three antibodies that reacted with the glycoprotein G and one antibody that reacted with the NP (58,177). Two of the antibodies reacted selectively with subgroup A (B90 and B109), three antibodies reacted with group B (7858, 9177 and 8239) and one reacted with both groups (7901) (58, 179). RS virus infected GMK-cells were washed with versenate and containing 1% trypsin and the cells were collected after centrifugation at 2000 rpm for 10 min. After centrifugation trypsin was removed and the cell pellet was suspended in 2 ml MEM and centrifuged at 2000 rpm for 10 min. Most of the medium was removed and the pellet was mixed with a small volume of remaining medium. Twenty µl of every specimen was dropwise dispensed onto small circumscribed areas on glass slides. The samples were air

dried and fixed with cold 100 % acetone for 10 min. Twenty-five µl of monoclonal antibodies diluted 1:50 in phosphate-buffered-saline (PBS), PH 7,2 was added. After incubation at 37°C for 30 min, the glass was washed in FA-buffer with 0,005% tween 20, PH 7,2. Fluorescein conjugated rabbit anti-mouse immunoglobuline diluted 1:15 in PBS in a volume of 25 µl was dispensed onto the samples after which they were incubated at 37°C for 30 min. After another washing the specimens were counterstained with Evans Blue at a final dilution of 1/3000 in distilled water for 2-3 min. After that the glass was washed in distilled water and mounted with 70% glycerol in veronal buffer, pH 8,6 and analyzed with a Leitz fluorescence microscope.

6.2.4 Probe design and melt curve analysis (paper III)

Following completion of the RT-PCR, melting curve analysis was achieved using two pairs of FRET hybridization probes (A and B) consisting of an indicator probe and a reporter probe (Paper III, Table 1). The indicator probes were labeled with fluorescein at the 3' end and was designed to hybridize directly over the selected regions of polymorphism. They were further designed with a bias for better complementarity to GA7 and GA5, respectively, with the aim to maximize melting temperature differences in the assay. The reporter probes, labelled at the 5' end with Cy5, were designed to anneal in direct proximity to the indicator probes. The reporter probes were designed to be more thermodynamically stable than the indicator probes to ensure that the probe remained hybridized during the melting step of the assay. Further, they were also biotinylated in the 3' end to prevent reporter probes from acting as a primers. The two pairs of FRET-probes were added to individual aliquots of all isolates after completion of the RT-PCR to a final concentration of 400 nmol/L. Subsequently, the samples were heated to 95°C for 1 minute followed by a decrease in the temperature to 32°C allowing for probe hybridization to occur. The following melting step consisted of a slow increase in temperature until a temperature of 95°C was obtained. Fluorescence was measured at 660 nm at every 1°C increase in temperature. The two T_m determinations obtained for each of the isolates were plotted in a two-dimensional system (Paper III, Fig.1). PCR and the melting reaction were performed in a RotorGene 2000 from Corbett Research, NSW; Australia. Results were analysed using the Corbett RotorGene software version 6.0. All oligonucleotides were purchased from Thermohyba (Ulm, Germany).

7 RESULTS AND DISCUSSION

7.1 Study on mumps virus neurovirulence (paper I)

During an epidemic in Lithuania in 1998-2000 isolated mumps virus strains were studied. Viruses of the neurovirulent C1 and non-neurovirulent C2 small hydrophobic (SH) genotype variant were sequenced for the haemagglutinin-neuraminidase (HN) and fusion (F) protein genes. Amino acid differences between C1 and C2 strains were found for both proteins. Two amino acid differences were of potential importance for the non-neurovirulent phenotype of the C2 virus. Four of five C2 strains exhibited the amino acid arginine instead of lysine at position 335 of the HN protein, and the amino acid phenylalanine was found instead of serine at amino acid position 195 of the F protein. Amino acid differences at these positions have previously been reported to associate with a change in neurovirulence and fusion activity. In addition, the HN gene of the neurovirulent Kilham strain of genotype A was sequenced for the first time. The deduced amino acid sequence showed different amino acids compared to both genotypes A and C on some positions. Notably, amino acid differences located in previously identified neutralizing epitopes were found at positions 266, 354 and 356 of the HN protein compared to other genotype A strains. The amino acid differences between the Kilham virus strain and other genotype A strains and the similarity of the Kilham HN protein to neurovirulent genotype C strains on some amino acid positions may indicate a possible role for this protein in mumps virus neurovirulence.

The study shows that there exist pronounced amino acid differences even in virus strains belonging to the same genotype of virus. The C1 and C2 subtypes of genotype C were different in both clinical symptoms and amino acids in many positions. However, the biological significance of the different amino acid changes is difficult to evaluate. Except for the mutation of serine to phenylalanine in position 195 of the F protein in C2 strains, no amino acid differences were found that would be expected to change the tertiary structure of the proteins. The potential N glycosylation sites were preserved in both proteins and there were no substitutions of the amino acid cysteine in either C1 or C2. Further in vivo and in vitro studies seem important in order to compare the biological properties of the two virus variants. In the case that future studies show that the attenuated phenotype of C2 virus is genetically stable, this virus may become a potential vaccine candidate for vaccination against mumps.

An interesting result was the finding that there existed many amino acid differences between the HN protein of the Kilham strain of genotype A compared to known genotype A strains.

Amino acid differences were found in a neutralising epitope of mumps virus (amino acid positions 352 to 360) between Kilham and genotype A and C. In the Kilham virus strain histidine and serine were found in the 354th and 356th positions, whereas both genotype A and C have different amino acids in these positions, proline and glutamic acid in genotype A and glutamine and aspartic acid in genotype C, respectively.

The A genotype is serologically distinct from genotype C. The amino acid differences between the Kilham virus strain compared to other genotype A strains and to the C genotype may provide an explanation for the type-specific behaviour of the Kilham virus found in neutralization assays. It is known that the Kilham strain exhibits a high neurovirulence. In this respect it differs from other genotype A strains which exhibit low neurovirulence.

Except for the C2 variant, the C genotype exhibits a high neurovirulence. The resemblance of the HN protein of Kilham strain to genotype C strains and the differences of the Kilham strain compared to other known strains of genotype A especially at virus neutralisation epitopes, position 266, 354 and 356, may suggest that the HN protein has a possible role in mumps virus neurovirulence. In spite of numerous efforts in research a common denominator for mumps neurovirulence has not yet been found.

7.2 Study on the molecular epidemiology of RSV in Stockholm (paper II)

The epidemiology of respiratory syncytial virus (RSV) group A was followed by nucleotide sequencing of the variable parts of the glycoprotein (G) gene. The amino acid sequences of an amino-terminal (NH₂-terminal, amino acids 90–132) and carboxy-terminal (COOH-terminal, amino acids 262–298) portion of the G protein in 47 virus strains, collected in Stockholm, between 1965 and 2004, were determined. In phylogenetic analysis jointly with previously described genotypes (GA1 to GA7 and SAA1), 34 virus strains (isolated between 1991 and 2004) belonged to genotype GA5, seven to GA2, three to genotype GA1 (isolated before 1991), one to genotype GA4 (isolated in 1982) and two to genotype GA7 (isolated in 1993 and 2001). Genotype GA5 was predominant in four epidemics, between 2000/2001 and 2003/2004 (Table 1). Little or no variation with time of the C-terminal amino acid sequence of the G protein was found when the virus strains were compared within their own genotype. Identical and nearly identical nucleotide sequences were found between strains isolated more than 10 (GA5) and 25 (GA2) years apart. The N-terminal part of the G protein of genotype

GA2 was highly conserved. In contrast, the N-terminal part of the G protein of genotype GA5 exhibited a pronounced variation in its amino acid sequence over time.

Year	No. of cases	Genotypes				
		GA1	GA2	GA4	GA5	GA7
1965	1	1				
1976	1		1			
1982	2	1		1		
1990	1	1				
1991	2				2	
1993	4		1		1	2
1994	1				1	
1996	1		1			
1997	1				1	
2000	1				1	
2001	8		1		7	
2002	10				10	
2003	8		2		6	
2004	6		1		5	
Total	47	3	7	1	34	2

Table 1. Determination of the genotype of 47 RSV group A collected isolated in Stockholm between 1965 and 2004

One could speculate that there exists a functional relationship between high variation in the N-terminal part of the protein and the high number of isolates of the GA5 genotype. In that, the variation in the A-terminal part of the protein of GA5 may affect its antigenicity, and thereby, make it possible for the virus to evade the immune system of the host. For example, serine at amino acid position 117 has been predicted to be *O*-glycosylated. In the case that the virus strain loses its *O*-glycosylation, it may escape the immune system by losing a carbohydrate epitope. It was found in many GA5 strains that serine in amino acid position 117 had changed to amino acid leucine or proline, but in all the seven GA2 strains serine was present in this position.

The variation of genotypes between different RSV epidemics has been explained by the gradual built up of immunity in the population against a certain virus genotype, allowing new genotypes to appear in subsequent epidemics. This does not appear to have happened in Stockholm as the GA5 genotype has dominated for at least four epidemic seasons. One may speculate about the possibility that the variation in the N-terminal part of the protein may

have allowed prolonged dominance of the genotype, but at present, there is lack of proof to support such a hypothesis. From previous studies, the importance of the C-terminal part of the protein for immunity has been demonstrated both in animals and humans, but the biological function of the N-terminal variable part of the G protein in immunity is still unknown. In spite of the fact that neutralising antibodies are formed against the C-terminal variable part, the nucleotide and amino acid sequence showed very little divergence over time within each genotype that was studied in the present investigation. There is a variation between the genotypes, but within each genotype, the amino acid sequence of the C-terminal part was remarkably conserved over long periods of time.

According to a study of the epidemiology of RSV in Stockholm during one season (2002-2003), groups A and B were co-circulating, with a dominance of subgroup B [182]. By sequencing the N-terminal of the G gene it was shown that closely related RSV strains could be found in different parts of the world at the same time and that the same genotypes have been circulating in Europe for a long time [182].

These conclusions were similar to the conclusions in the present study.

7.3 To develop a novel method based on RT-PCR followed by two-dimensional melting curve analysis based on FRET (paper III)

Eighty RSV isolates were obtained from patients with respiratory tract infection (Table 2). Thirty samples were from a previous study [183] and fifty samples were collected in the 2000/2001 to 2004/2005 epidemic seasons, representing three genotypes of RSV; GA2, GA5 and GA7. The genotype distribution of all tested isolates was 10 GA2, 64 GA5 and six GA7 (Table 2). The results from genotyping by nucleotide sequencing and melting curve analysis for all samples can be seen in Fig.1 (paper III). The melting temperatures for 89% (71/80) of the samples were clustered in defined temperature intervals (cf. boxes A, B and C in Fig.1 (paper III) and represented GA2, GA7 and GA5 respectively). However, nine samples, belonging to GA2 and GA5, showed melting temperatures that differed from most of the isolates belonging to the same genotype (boxes A and C). These samples were regarded as untypeable. For some of the samples an altered pattern of nucleotide polymorphism within the target sequence of the probes correlated with a deviant melting temperature.

The mean T_m and standard deviation ($^{\circ}\text{C}$) for respective genotypes (GA2, GA5 and GA7) were 50.7 ± 2.5 , 46.0 ± 3.1 and 62.4 ± 0.5 (A probes) and 49.9 ± 4.3 , 61.6 ± 4.9 and 54.0 ± 3.1 (B probes).

Year	No. of cases	Genotypes		
		GA2	GA5	GA7
1976	1	1		
1991	2		2	
1993	4	1	1	2
1994	1		1	
1997	1		1	
2000	1		1	
2001	15	4	9	2
2002	18		18	
2003	13	1	12	
2004	15	1	14	
2005	9	2	5	2
Total	80	10	64	6

Table 2. Genotyping distribution of the eighty RSV group A virus isolated collected in Stockholm between 1976 and 2005

This and the previous study [183] show that the GA5 genotype has been the dominating genotype in Stockholm during the last five epidemic seasons (Table 2). Fifty-eight out of 71 isolates (82%) were found to belong to GA5 during this time, eight were GA2 and four were GA7.

From the results presented in Fig.1 (paper III) it was concluded that 89% of the Swedish material could be genotyped exclusively on the basis of nucleotide polymorphism in the target area of the probes. Some strains had nucleotide polymorphisms different from the other samples of the same genotype, which could explain their unexpected T_m values. Obviously, one weakness of the present study regarding the generality of the method is the lack of GA1 and GA4 genotypes in studied samples. The GA1 and GA4 genotypes were not found in Stockholm after 1990 and 1982, respectively. Owing to the lack of sufficient numbers of GA1 and GA4 genotypes, the present investigation was based on three genotypes (GA2, GA5 and GA7), which have been circulating in Stockholm during the past decade. Owing to the

sequence diversity and expected continuous sequence changes of the G protein of the RS virus, it is expected that sequence modifications will need to be introduced in probes to obtain optimal separation of future variants. Resolution of genotypes can also be expected to increase through introduction of a third FRET probe.

The proposed model for interpretation of results will identify nontypeable samples for sequencing, hence reducing sequencing efforts. Two-dimensional plotting of data has been applied in many fields to enhance resolution and can be expected to be generally applicable to any genetic typing systems where two or more polymorphic regions are accessible for T_m or other discriminating determinations.

7.4 Study on epidemiology of hMPV in Stockholm (paper IV)

The presence of hMPV was retrospectively investigated in 4,989 samples, 2,659 samples negative for RSV, influenza A and B, parainfluenza and adenovirus and 2,330 samples, positive for these respiratory viruses.

The detection results showed an average incidence of 2,9% (n = 143/4,989) in five seasons [2,0%, n = 19/933 (2001/2002); 5,9%, n = 51/870 (2002/2003); 0,8%, n = 9/1069 (2003/2004); 4,8%, n = 49/1027 (2004/2005) and 1,4%, n = 15/1090 (2005/2006)] by RT-PCR (Paper IV, Figure 1A, Table 1). The hMPV detection in samples negative for other respiratory tract viruses was 5,4% (n = 143/2569). A higher number of hMPV-positive samples were found in the 2002/2003 season followed by the 2004/2005 season as compared to the other three seasons (Paper IV, Figure 1A, Table 1).

Based on genomic sequencing and phylogenetic analysis app. 96% (87/91) of all hMPV sequenced strains belonged to genotype A, and 4% (4/91) belonged to genotype B (paper IV, Figure 5). Out of four genotype B viruses two were from patients younger than 3 years of age and two were from patients older than 3 years. Genotype B was not found after the season 2003/2004.

No co-infections cases were found in the material between hMPV and the other respiratory viruses. Four other co-infections were found in the material. Two were with influenza A viruses and RSV, one with influenza A and parainfluenza virus and one with adenovirus and RSV. Human metapneumovirus (hMPV) was most frequently detected in March and followed by February (Paper IV, Figure 3). For example, hMPV incidence peaked in March, 2002/2003 (14,7%, n=21/143), followed by February-March 2004/2005 (11,9%, n=17/143). During the

seasons 2001/2002, 2002/2003 and 2003/2004 hMPV was detected mostly in March, and the season 2004/2005 it was detected mostly in February – March. The last season 2005/2006, it was detected mostly in April and May, later than in the other seasons. The late seasonal peak of hMPV infection, compared to RSV followed in four of the five seasons.

Large hMPV virus epidemics occurred biannually and were anticyclical to RSV epidemics.

In the present study from Stockholm, Sweden, hMPV was less frequently detected than RSV, influenza A virus and parainfluenza viruses.

Since the discovery of hMPV in 2001, the virus has been found worldwide and the frequency of hMPV detection in patients with respiratory tract infection ranged from 1,5 to 43% in one study [184]. The various incidence rates of human pneumovirus in respiratory infections in different studies may be due to different study design. Several factors such as seasons, type of samples, detection method and age may have contributed to this difference. Studies on the incidence of hMPV from other European countries have reported both lower and higher figures compared to the present study from Sweden.

It was found that hMPV-infected children were significantly older than RSV-infected children in accordance with the results from other studies. The results from the present study showed that metapneumovirus and parainfluenza frequencies were anticyclical with respiratory syncytial virus [185]. Differences in the frequency of hMPV, PIV and RSV infections have been found every two years. In the seasons 2001/2002, 2003/2004 and 2005/2006 the frequencies of hMPV and PIV infections were low but with high frequency of RSV. In the seasons 2002/2003 and 2004/2005 the frequency of hMPV and PIV were high, but low frequency of RSV was found. The same finding has recently been reported by German researchers [185]. Although the reasons for this phenomenon are not known at present, these findings should have a significant effect on diagnostics and on the epidemiologic studies of infections with respiratory viruses. It is well known for RSV that large epidemics are followed by small epidemics every second year. The same phenomenon appears to occur also for hMPV and parainfluenza viruses.

HMPV strains were separated into genotypes A and B. A marked dominance of genotype A was found. During the 5 seasons under study the A genotype dominated in both age groups. Out of 91 sequenced samples, 52 belonged to patients younger than 3 years. Boivin et al have reported that the group A genotype infects three times as many children ≤ 3 years of age. In addition, group B strains occurred more frequently in adults [186]. In the present study genotype A was dominant in both age groups. The different results in the two studies may be

due to dynamic differences in the occurrence and fluctuations of the two genotypes in different countries/continents.

In agreement with several other studies hMPV activity was found with a higher frequency in late winter and most in spring [187]. This seasonal distribution is similar for 5 consecutive seasons in study, 2002-2006. The strong peak in the circulation of hMPV in the spring has distinguished hMPV from the other respiratory tract viruses especially from RSV. However, a few studies have shown that in isolated seasons the peak of hMPV epidemics may occur in December and January together with RSV [188,189].

It was concluded that the epidemiology of hMPV differs markedly from other common respiratory viruses.

8 CONCLUSIONS

- Amino acid differences between C1 and C2 strains of mumps virus were found for two envelope glycoproteins, F and HN.
- The amino acid differences between Kilham virus strain and other genotype A strains and the similarity of the Kilham HN protein to neurovirulent genotype C strains on some amino acid positions may indicate a possible role for this protein in mumps virus neurovirulence.
- The data presented from Stockholm demonstrated a dominance of RSV GA5 genotype strains in five consecutive seasons followed by GA2 genotype.
- It was found that the new assay (RT-PCR followed by two-dimensional melting curve analysis based on FRET) was able to make correct genotype identification in about 89% of the isolates of RSV and identified the remaining 11% as untypeable and candidates for conventional nucleotide sequencing.
- It was concluded that the epidemiology of hMPV differs markedly from other common respiratory viruses.

9 Populärvetenskaplig sammanfattning

Avsikten med avhandlingen är att öka kunskapen om epidemiologiska och strukturella egenskaper hos några medlemmar ur virusfamiljen paramyxovirus. En större kunskap inom dessa områden kan leda till nya och förbättrade vacciner i framtiden. I familjen paramyxovirus ingår flera humanpatogena virustyper som ofta drabbar barn, varför flera av dessa virusorsakade sjukdomar går under benämningen barnsjukdomar. Paramyxovirus uppdelas i två subfamiljer, paramyxovirus, några vanliga medlemmar inom denna grupp är påssjukevirus och mässlingsvirus, samt pneumovirus representerade av respiratoriskt syncytievirus samt humant metapneumovirus. I denna avhandling studeras påssjukevirus, respiratoriskt syncytievirus (eng. respiratory syncytial virus) och humant metapneumovirus.

Påssjukevirus ger upphov till påssjuka, namnet syftar på att spottkörtlarna förstöras, men virus kan också spridas till andra organ. Virusinfektionen är framför allt en viktig orsak till hjärnhinneinflammation (meningit). Påssjukevirus består i dag av tretton olika genotyper (benämnda från A till M) baserade på olikheterna hos en liten gen i virushöljet, den s.k. "small hydrophobic" (SH) genen. Två viktiga frågeställningar inom påssjukevirologin är varför en del vacciner ger ett ofullständigt skydd och varför vissa virusvarianter till skillnad från andra ger upphov till meningit hos den drabbade individen.

Tidigare studier på genotyp C från en epidemi i Litauen år 1998-2000 visade att dessa stammar gav upphov till parotit med eller utan meningit. Genotyp C var tidigare känd som orsak till meningit. De stammar som endast gav upphov till parotit uppvisade två specifika aminosyramutationer i SH proteinet kodat av genen. Studien i avhandlingen av två andra gener, fusions (F) och hemagglutinin-neuraminidas (HN) genen, förstärkte hypotesen att det finns systematiska skillnader mellan de två virusvarianterna. Resultaten kan leda till ett nytt förslag till två subgrupper av genotyp C, neuropatogen C1 (tidigare C) och icke neuropatogen C2.

Respiratoriskt syncytievirus (RS virus) ger upphov till luftvägsinfektioner som huvudsakligen drabbar små barn. Infektioner orsakade av detta virus är den vanligaste orsaken till nedre luftvägsinfektioner hos spädbarn. Återinsjuknande är vanligt förekommande både hos barn och vuxna, vanligtvis förenat med lindriga symptom. Stora epidemier förekommer vinter och vår. Infektioner orsakade av RS virus kan vara livshotande när de drabbar spädbarn. Det finns ett behov av att vaccinera personer med benägenhet att drabbas av svåra RS virus infektioner, nämligen barn under två år, äldre och immunsupprimerade personer, men ännu finns inget

vaccin tillgängligt. Svårigheterna omfattar exempelvis variabilitet av virus antigena egenskaper, virusets förmåga att åstadkomma upprepade infektioner under livstiden samt virusets förmåga att orsaka sjukdomar hos spädbarn trots närvaro kvardröjande antikroppar hos barnet från modern (s.k. maternella antikroppar). RS virus indelas i två grupper, A och B, främst baserade på antigena skillnader hos glykoproteinet (G proteinet) i virushöljet. RS virus grupp A har rapporterats innehålla åtta olika genotyper, GA1-GA7 samt SAA1. Med molekylärbiologiska metoder studerades RS virus epidemiologi under en längre tidsperiod (1965-2005). Genotyp GA5 dominerade i antal under de fem senaste luftvägssäsongerna, från säsongen 2000/2001 till 2004/2005 och GA2 var den näst vanligaste genotypen. Dessa resultat överensstämmer med fynd från övriga delar av världen. Ett intressant fynd är att genotyp GA1 dominerat i världen fram till 1990 och därefter helt försvunnit. I samband med dessa studier gjordes försök att åstadkomma en enklare metod för genotypning med realtids PCR-FRET (Fluorescence Resonance Emission Transfer) och smältpunktsanalys med hjälp av ett speciellt instrument. Försöken ledde till slutsatsen att denna metod i sin nuvarande utformning inte förmådde ersätta nukleotidsekvensering för genotypning av RS virus.

Humant metapneumovirus (hMPV) som upptäcktes av holländska forskare år 2001 ger upphov till en liknande sjukdomsbild som RS virus. Undersökning av arkiverade blodprov har visat att viruset funnits under en lång tid men förblivit oupptäckt, sannolikt beroende på att det är relativt ovanligt i jämförelse med RS virus samt kräver specifika betingelser för isolering i cellkulturer. Viruset delar flera genetiska egenskaper med RS virus, inklusive uppdelningen i två grupper, A och B, baserade på skillnader i G proteinet. Varje grupp har två undergrupper, subgrupp 1 och 2. Det är oklart om de två grupperna utgör två serologiskt skilda virus. Den molekylärepidemiologiska studien av hMPV i avhandlingen sträckte sig över en längre tidsperiod och visade att hMPV var mindre vanligt förekommande än RS virus, influensa A och parainfluensavirus. Åldersdistributionen i patientmaterialet skiljde sig dessutom från dessa virus. I den svenska studien dominerade grupp A över grupp B. Det viktigaste resultatet i studien var att större epidemier av hMPV inträffar vart annat år och förekommer anticykliskt med stora RS virus epidemier. Ur flera epidemiologiska aspekter skiljer sig hMPV från andra vanliga luftvägsvirus.

Sammanfattningsvis har denna avhandling studerat (i) sekvenser av HN och F gener för C1 och C2 varianter av genotyp C. En typrepresentant av genotyp A, Kilham virus, med neuropatogen förmåga visade vissa likheter med genotyp C avseende HN genen., (ii)

molekylär epidemiologi av RS virus grupp A i Stockholm (iii) möjligheten av en alternativ genotypningsmetod av RS virus grupp A med hjälp av realtids PCR:FRET samt studerat (iv) jämförande epidemiologi av hMPV och andra vanliga luftvägsvirus i samtliga åldersgrupper av patienter.

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11 REFERENCES

1. Weibel R. Mumps vaccines. In: Plotkin SA, Moritimer Jr EA, eds. Vaccines Philadelphia: 1988; 223-234.
2. Plotkin SA, Wharton M. Mumps vaccine. In: Plotkin SA, Moritimer Jr EA, eds. Vaccines, 3rd. Philadelphia: WB Saunders, 1999; 267-292.
3. Hamilton R. An account of a distemper by the common people of England vulgarly called the mumps. London Med Journal. 1970; 11:190-211.
4. Johnson CD, Goodpasture EW. The etiology of mumps. Am. Journal Hyg. 1935; 21: 46-57.
5. Habel K. Cultivation of mumps virus in the developing chick embryo and its application to studies of immunity to mumps in man. Public Health Rep. 1945; 60:201-212.
6. Levens JH, Enders JF. The hemagglutinative properties of amniotic fluid from embryonated eggs infected with mumps virus. Science. 1945; 102:117-120.
7. Morgan HR, Enders JF, Wagley PF. A homolysin associated with the mumps virus. Journal of Exp Med. 1948; 88:503-514.
8. Henle G, Deinhardt F. Propagation and primary isolation of mumps virus in tissue cultures of epithelial cells. Proc. Soc Exp Biol Med. 1955; 89:556-560.
9. Bennett AD, Modlin J, Orenstein WA, Brandling. Current status of mumps in the United States. J Infect Dis. 1975; 132:106-109.
10. Centers for Disease Control and Prevention. Summary of notifiable disease, 1992. MMWR Mor Wkly Rep. 1993; 41: iv -73.
11. Centers for Disease Control and Prevention. Summary of notifiable disease, 1998. Mor MMWR Mor Wkly Rep. 1999; 47:1123.
12. Blount RE, Morris JA, Savage RE. Recovery of cytopathic agent from chimpanzee with coryza. Proc Soc Exp Biol Med. 1956; 92:544-550.
13. Chanock R, Roizman B, Myers R, Recovery from infants with respiratory illness of a virus related to chimpanzee coryza agent. I. Isolation, properties and characterization . Am J Hyg 1957; 66:281-290.
14. International conference on acute respiratory infection. Acute respiratory infections: The forgotten pandemic. Communique from the International conference on acute respiratory infections, held in Canberra, Australia, 7-10 July 1997. Int J Tuberc lung Dis. 1998; 2:2-4.
15. Gardner PS. Respiratory syncytial virus infection. Postgrad Med J. 1973; 49:788-791.

16. Glezen P, Denny FW. Epidemiology of acute lower respiratory disease in children. *N Engl J Med.* 1973; 288: 498-505.
17. Holberg CJ, Wright AL, Martinez FD, Ray CG, Taussig LM, Lebowitz MD. Risk factors for respiratory syncytial virus-associated lower respiratory illnesses in the first year of life. *Am J Epidemiol.* 1991; 33:1135-1151.
18. Kim HW, Arrobio JO, Brandt CD, Jeffries BC, Pyles G, Reid JL, Chanock RM, Parrott RH. Epidemiology of respiratory syncytial virus infection in Washington, DC.I. Importance of the virus in different respiratory tract disease syndromes and temporal distribution of infection. *Am J Epidemiol.* 1973; 98: 216-225.
19. Murphy BR, Prince GA, Collins PL, Van Wyke Coelingh K, Olmsted RA, Spriggs MK, Parrott RH, Kim HW, Brandt CD, Chanock RM. Current approaches to the development of vaccines effective against parainfluenza and respiratory syncytial virus. *Virus Res.* 1988; 11:1-15.
20. Parrott RH, Kim HW, Arrobio JO, Hodes DS, Murphy BR, Brandt CD, Camargo E, Chanock RM. Epidemiology of respiratory syncytial virus infection in Washington, DC. II. Infection and disease with respect to age, immunologic status, race and sex. *Am J Epidemiol.* 1973; 98: 289-300.
21. Falsey AR, Treanor JJ, Betts RF, Walsh EE. Viral respiratory infections in the institutionalized elderly; Clinical and epidemiologic findings. *J Am Geriatr Soc.* 1992; 40: 115-119.
22. Han LL, Alexander JP, Anderson LJ. Respiratory syncytial virus pneumonia among the elderly: An assessment of disease burden. *J Infect Dis* 1999; 179: 25-30.
23. Wendt CH, Hertz MI. Respiratory syncytial virus and parainfluenza virus infections in the immunocompromised host. *Semin Respir Infect.* 1995; 10:224-231.
24. Whimbey E, Champlin RE, Couch RB, Englund JA, Goodrich JM, Raad I, Przepiorka D, Lewis VA, Mirza N, Yousef H, Tarrand JJ, Bodey GP. Community respiratory virus infections among hospitalized adult bone marrow transplant recipients. *Clin Infect Dis.* 1996; 22: 778-782.
25. Murry AR, Dowell SF. Respiratory syncytial virus: Not just for kids. *Host Pract.* 1997; 32: 87-88, 91-84, 98 passim.
26. Falsey AR, Walsh EE. Respiratory syncytial virus infection in adults. *Clin Microbiol Rev.* 2000;13: 371-384.
27. Kahn JS. Respiratory syncytial virus vaccine development. *Curr Opin Pediatr.* 2000; 12: 257-262.

28. Murphy BR, Prince GA, Walsh EE, Kim HW, Parrott RH, Hemming VG, Rodriguez WJ, Chanock RM. Dissociation between serum neutralizing and glycoprotein antibody responses of infants and children who received inactivated respiratory syncytial virus vaccine. *J Clin Microbiol.* 1986; 24: 197-202.
29. Kim HW, Canchola JG, Brandt CD, Pyles G, Chanock RM, Jensen K, Parratt RH. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. *Am J Epidemiol.* 1969; 89: 422-434.
30. Kapikian AZ, Mitchell RH, Chanock RM, Shvedoff RA, Stewart CE. An epidemiologic study of altered clinical reactivity for respiratory syncytial (RS) virus infection in children previously vaccinated with an inactivated RS virus vaccine. *Am J Epidemiol.* 1969; 89: 405-421.
31. Fulginiti VA, Eller JJ, Sieber OF, Joyner JW, Minamitani M, Meiklejohn G. 1969. Respiratory virus immunization. I. A field trial of two inactivated virus vaccines, an aqueous trivalent parainfluenza virus vaccine and an alum-precipitated respiratory syncytial virus vaccine. *Am J Epidemiol.* 1969; 89: 435-438.
32. Chin J, Magoffin RL, Shearer LA, Schieble JH, Lennette EH. Field evaluation of a respiratory syncytial virus vaccine and a trivalent parainfluenza virus vaccine in a pediatric population. *Am J Epidemiol.* 1969; 89:449-463.
33. van den Hoogen BG, de Jong JC, Groen J, Kuiken T, de Groot R, Fouchier RA, Osterhaus ADME. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. *Nat Med.* 2001; 7:719-724.
34. Boivin G, Abed Y, Pelletier G, Ruel L, Moissan D, Cote'S, Peret TC, Erdman DD, Anderson LJ. Virological features and clinical manifestations associated with human metapneumovirus: A new paramyxovirus responsible for acute respiratory-tract infections in all age groups. *J Infect Dis.* 2002; 186:1330-1334.
35. Boivin G, De Serres G, Cote'S, Gilca R, Abed Y, Rochette L, Bergeron MG, Dery P. Human metapneumovirus infections in hospitalized children. *Emerg Infect Dis.* 2003; 9: 634-640.
36. Esper F, Boucher D, Weibel C, Martinello RA, Kahn JS. Human metapneumovirus infection in the United States: Clinical manifestations associated with a newly emerging respiratory infection in children. *Pediatrics.* 2003; 111: 1407-1410.
37. Chan PK, Tam JS, Lam CW, Chan E, Wu A, Li CK, Buckley TA, Ng KC, Joynt GM, Cheng FW, To KF, Lee N, Hui DS, Cheung JL, Chu I, Liu E, Chung SS, Sung JJ . Human

- metapneumovirus detection in patients with severe acute respiratory syndrome. *Emerg Infect Dis.* 2003; 9:1058-1063.
38. Freymuth F, Vabret A, Legrand L, Etteradossi N, Lafay-Delaire F, Brouard J, Guillois B. Presence of the new human metapneumovirus in French children with bronchiolitis. *Pediatr Infect Dis J.* 2003; 22: 92-94.
 39. Vicenti D, Cilla G, Montes M, Perez-Trallero E. Human metapneumovirus and community-acquired respiratory illness in children. *Emerg Infect Dis.* 2003; 9:602-603.
 40. Osterhaus A, Fouchier R. Human metapneumovirus in the community. *Lancet.* 2003; 361:890-891.
 41. McIntosh K, McAdam AJ. Human metapneumovirus--an important new respiratory virus. *N Engl J Med.* 2004; 350:431-433.
 42. Lamb RA, Kolakofsky D. Paramyxoviridae: The viruses and their replication. *Fields 4th*. Philadelphia: 2001; 1305-1340.
 43. Rima BK, Alexander DJ, Billeter MA, Collins PL, Kingsbury DW, Lipkind MA, Nagai Y, Örvell C, Pringle C, Ter Meulen V. The Paramyxoviridae. In *virus Taxonomy*. Sixth report of the international committee on taxonomy of viruses. 1995; 268-274.
 44. Elango N, Varsanyi TM, Kövamees J, Norrby E. Molecular cloning and characterization of six genes, determination of gene order and intergenic sequences and leader sequence of mumps virus. *J Gen Virol.* 1988;69: 2893-2900.
 45. Amexis G, Rubin S, Chizhikov V, Pelloquin F, Carbone K, Chumakov K. Sequence diversity of Jeryl Lynn strain of mumps virus: quantitative mutant analysis for vaccine quality control. *Virology.* 2002; 300(2):171-179.
 46. Waxham MN, Server AC, Goodman HM, Wolinsky JS. Cloning and sequencing of the mumps virus fusion protein gene. *Virol.* 1987; 159:381-388.
 47. Waxham MN, Aronowski J, Server AC, Wolinsky JS, Smith JA, Goodman HM. Sequence determination of the mumps virus HN gene. *Virology.* 1988; 164: 318-325.
 48. Elliott GD, Afzal MA, Martin S, Rima BK. Nucleotide sequence of the matrix, fusion and putative SH protein genes of mumps virus and their deduced amino acid sequences. *Virus Res.* 1989; 12(1):61-75.
 49. Kövamees J, Norrby E, Elango N. Complete nucleotide sequence of the hemagglutinin-neuraminidase (HN) mRNA of mumps virus and comparison of paramyxovirus HN proteins. *Virus Res.* 1989; 12(1):87-96.
 50. Elango N. The mumps virus nucleocapsid mRNA sequence and homology among the Paramyxoviridae proteins. *Virus Res.* 1989; 12(1):77-86.

51. Takeuchi K, Hishiyama M, Yamada A, Sugiura A. Molecular cloning and sequence analysis of the mumps virus gene encoding the P protein: mumps virus P gene is monocistronic. *J Gen Virol.* 1988; 69:2043-2049.
52. Takeuchi K, Tanabayashi K, Hishiyama M, Yamada A. The mumps virus SH protein is a membrane protein and not essential for virus growth. *Virology.* 1996; 225(1):156-62.
53. Inou Y, Nakayama T, Yoshida N, Uejima H, Yuri K, Kamada M, Kumagai T, Sakiyama H, Miyata A, Ochiai H, Ihara T, Okafuji T, Nagai T, Suzuki E, Shimomura K, Ito Y, Miyazaki C. Molecular epidemiology of mumps virus in Japan and proposal of two new genotypes. *J Med Virol.* 2004; 13: 97-104.
54. Jin L, Brown DW, Litton PA, White JM. Genetic diversity of mumps virus in oral fluid specimens: application to mumps epidemiological study. *J Infect Dis.* 2004; 189: 1001-1008.
55. Santos CLS, Ishida MA, Foster PG, Sallum MAM, Benega MA, Borges DB, Correa KO, Constantino CRO, Afzal MA, Paiva TM. Detection of a new mumps virus genotype during parotitis epidemic of 2006-2007 in the state of Sao Paulo, Brazil. *J Med Virol.* 2008; 80: 323-329.
56. Collins PL, Crowe JEJ. History and classification. *Fields 5th*. Philadelphia: 2007; 16601-1646.
57. Anderson LJ, Hierholzer JC, Tsou C, Hendry RM, Fernie BN, Stone Y, McIntosh K. Antigenic characterization of respiratory syncytial virus strains with monoclonal antibodies. *J Infect Dis.* 1985; 151:626-633.
58. Mufson MA, Örvell C, Rafnar B, Norrby E, Two distinct subtypes of human respiratory syncytial virus. *J Gen Virol.* 1985; 66:2111-2124.
59. Peret TC, Hall CB, Schnabel KC, Golub JA, Anderson LJ. Circulation patterns of genetically distinct group A and B strains of human respiratory syncytial virus in a community. *J Gen Virol.* 1998; 9:2221-2229.
60. Venter M, Madhi SA, Tiemessen CT, Schoub BD. Genetic diversity and molecular epidemiology of respiratory syncytial virus over four consecutive seasons in South Africa: identification of new subgroup A and B genotypes. *J Gen Virol.* 2001; 82: 2117-2124.
61. Venter M, Collinson M, Schoub BD. Molecular epidemiology analysis of community circulating respiratory syncytial virus in rural South Africa: comparison of viruses and genotypes responsible for different disease manifestations. *J Med Virol.* 2002; 68: 452-461.

62. Frabasile S, Delfaro A, Facal L, Videla C, Galiano M, de Sierra MJ, Ruchansky D, Vitureira N, Berois M, Carballal G, Russi J, Arbiza J. Antigenetic and genetic variability of human respiratory syncytial virus (group A) isolated in Uruguay and Argentina: 1993-2001. *J Med Virol.* 2003 71: 305-312.
63. van den Hoogen BG, Bestebroer TM, Osterhaus AD, Fouchier RA. Analysis of the genomic sequence of a human metapneumovirus. *Virology.* 2002; 295:119-32.
64. Mackay IM, Bialasiewicz S, Waliuzzaman Z, Chidlow GR, Fegredo DC, Laingam S, Adamson P, Harnell GB, Rawlinton W, Nissen MD, Slotts TP. Use of the P gene to genotype human metapneumovirus identifies 4 viral subtypes. *J Infect Dis* 2004; 190:1913-18.
65. Chambers P, Pringle CR, Easton AJ. Heptad repeat sequences are located adjacent to hydrophobic regions in several types of virus fusion glycoproteins. *J Gen Virol.* 1990; 71: 3075-3080.
66. Gordon JE, Kilham L. Ten years in the epidemiology of mumps. *Am J Med Sci.* 1949; 218: 338-359.
67. Roizman B. Multiplication of viruses: an overview. In Fields BN, Knipe DM, Howley PM eds. *Fields Virology*, 3rd Ed. Lippincott-Raven, Philadelphia. 1243.
68. Kilham L. Isolation of mumps virus from the blood of a patient. *Proc Soc Exp Biol Med.* 1948; 69:99-100.
69. Chiba Y, Horino K, Umetsu M, Wataya Y, Chiba S. Virus excretion and antibody response in saliva in natural mumps. *Tohoku J Exp Med.* 1973; 111:229-238.
70. Perry KR, Brown DW, Parry JV, Panday S, Pipkin C, Richards A. Detection of measles, mumps, and rubella antibodies in saliva using antibody capture radioimmunoassay. *J Med Virol.* 1993; 40:235-240.
71. Fleischer B, Kreth HW. Mumps virus replication in human lymphoid cell lines and in peripheral blood lymphocytes: Preference for T cells. *Infect Immun.* 1982; 35:25-31.
72. Wolinsky JS, Klassen T, Baringer JR. Persistence of neuroadapted mumps virus in brains of newborn hamsters after intraperitoneal inoculation. *J Infect Dis.* 1976; 133:260-267.
73. Johnson KM, Chanock RM, Rifkind D, Kravetz HM, Knight V. Respiratory syncytial virus. IV. Correlation of virus shedding, serologic response, and illness in adult volunteers. *JAMA.* 1961; 176:663-667.
74. Kapikian AZ, Bell JA, Mastropa FM, Johnson KM, Huebner RJ, Chanock RM. An outbreak of febrile illness and pneumonia associated with respiratory syncytial virus infection. *Am J Hyg.* 1961; 74:234-248.

75. Gardner PS, Mcquillin J, Court SD. Speculation on pathogenesis in death from respiratory syncytial virus infection. *Br Med J*. 1970; 1 327-330.
76. Neilson KA, Yunis EJ. Demonstration of respiratory syncytial virus in an autopsy series. *Pediatr Pathol*. 1990; 10:491-502.
77. Domurat F, Roberts NJ Jr, Walsh EE, Dagan R. Respiratory syncytial virus infection of human mononuclear leukocytes in vitro and in vivo. *J Infect Dis*. 1985; 152:895-902.
78. Bui RH, Molinaro GA, Kettering JD, Heiner DC, Imagawa DT, St Geme JW Jr. Virus-specific IgE and IgG4 antibodies in serum of children infected with respiratory syncytial virus. *J Pediatr*. 1987; 110: 87-90.
79. Welliver RC, Wong DT, Sun M, Middleton E Jr, Vaghan RS, Ogra PL. The development of Respiratory syncytial virus-specific IgE and the release of histamine in nasopharyngeal secretions after infection. *N Engl J Med*. 1981; 305:841-846.
80. Volovitz B, Welliver RC, DE Castro G, Krystofik DA, Ogra PL. The release of leukotrienes in the respiratory tract during infection with respiratory syncytial virus: Role in obstructive airway disease. *Pediatr Res*. 1988; 24:504-507.
81. Everard ML, Fox G, Walls AF, Quint D, Fifield R, Walters C, Swarbrick A, Milner AD. Tryptase and IgE concentrations in the respiratory tract of infants with acute bronchiolitis. *Arch Dis Child*. 1995; 72:64-69.
82. Toms GL, Quinn R, Robinson JW, Undetectable IgE responses after respiratory syncytial virus infection. *Arch Dis Child*. 1996; 74:126-130.
83. Grove JEJ. Human metapneumovirus as a major cause of human respiratory tract disease. *Pediatr Infect Dis J*. 2004; 23:215-221.
84. Jartti T, Van den Hoogen, Garofalo RP, Osterhause AD, Ruuskanen O. Metapneumovirus and acute wheezing in children. *Lancet*. 2002; 360:1393-1394.
85. Laham FR, Israele V, Casellas JM, Garcia AM, Lac Prugent CM, Hoffman SJ, Hauer D, Thumar B, Name MI, Pascual A, Taratutto N, Ishida MT, Balduzzi M, Maccarone M, Jackli S, Passarino R, Gaivironsky RA, Karron RA, Polack NR, Polack FP. Differential production of inflammatory cytokines in primary infection with human metapneumovirus and with other common respiratory viruses of infancy. *J Infect Dis*. 2004; 189:2047-2056.
86. Kuiken T, van den Hoogen BG, Van Riel DA, Laman JD, van Amerongen G, Sprong L, Fouchier RA, Osterhaus AD. Experimental human metapneumovirus infection of cynomolgus macaques (*Macaca fascicularis*) results in virus replication in ciliated epithelial cells and pneumocytes with associated lesion throughout the respiratory tract. *Am J Pathol*. 2004; 164:1893-1900.

87. Williams JV, Harris PA, Tollefson SJ, Halburnt-Rush LL, Pingsterhaus JM, Edwards KM, Wright FP, Crowe JE Jr. Human metapneumovirus and lower respiratory tract disease in otherwise healthy infants and children. *N Engl J Med.* 2004 350:443-450.
88. Ilonen J. Lymphocyte blast transformation response of seropositive and seronegative subject to herpes simplex, rubella, mumps and measles virus antigens, *Acta Pathol Microbiol Scand [C]*. 1979; 87:151-157.
89. Kupers TA, Petrich JM, Holloway AW, St Geme JW. Depression of tuberculin delayed hypersensitivity by live attenuated mumps virus. *J Pediatr.*1970; 76(5):716-721.
90. Bertotto A, Spinozzi F, Gerli R, Bassotti G, Forenza N, Pazzelli P, Bartolucci M, De Giorgi G, Vaccaro R. Gamma delta T lymphocytes in mumps meningitis patients. *Acta Paediatr.* 1995; 4(11):1268-1270.
91. Fleischer B, Kreth HW. Clonal analysis of HLA-restricted virus-specific cytotoxic T lymphocytes from cerebrospinal fluid in mumps meningitis. *J Immunol.* 1983; 130(5):2187-2190.
92. Kreth HW, Kress L, Kress HG, Ott HF, Eckert G. Demonstration of primary cytotoxic T cells in venous blood and cerebrospinal fluid of children with mumps meningitis. *J Immunol.* 1982; 128(6):2111-2415.
93. Truant AL, Hallum JV. Detection of mumps virus antigens in Hodgkin's disease tissue. *Oncology.* 1976; 33:241-245.
94. Wolinsky JS, Waxham MN, Server AC. Protective effects of glycoprotein-specific monoclonal antibodies on the course of experimental mumps virus meningoencephalitis. *J Virol.* 1985; 53(3):727-734.
95. Crow JE. Host responses to respiratory virus infection and immunization. *Curr Top Microbiol Immunol.* 1999; 236:191-214.
96. McIntosh K, Masters HB, Orr I, Chao RK, Barkin RM. The immunologic response to infection with respiratory syncytial virus in infants. *J Infant Dis.* 1978; 138:24-32.
97. Chiba Y, Higashidate Y, Suga K, Honjo K, Tsutsumi H, Ogra PL. Development of cell-mediated cytotoxic immunity to respiratory syncytial virus in human infants following naturally acquired infection. *J Med Virol.* 1989; 28:133-139.
98. Connors M, Collins PL, Firestone CY, Murphy BR. Respiratory syncytial virus (RSV) F,G,M2 (22K), and N proteins each induce resistance to RSV challenge, but resistance induced by M2 and N proteins is relatively short-lived. *J Virol.*1991; 65:1634-1637.
99. Gaddum RM, Cook RS, Wyld SG, Lopez JA, Bustos R, Melero JA, Taylor G.

- Mutant forms of the F protein of human respiratory syncytial (RS) virus induce a cytotoxic T lymphocyte response but not a neutralizing antibody response and only transient resistance to RS virus infection. *J Gen Virol.* 1996; 77:1239-48.
100. Connors M, Kulkarni AB, Collins PL, Firestone CY, Holmes KL, Morse HC 3rd, Murphy BR. Resistance to respiratory syncytial virus (RSV) challenge induced by infection with a vaccinia virus recombinant expressing the RSV M2 protein (Vac-M2) is mediated by CD8⁺ T cells, while that induced by Vac-F or Vac-G recombinants is mediated by antibodies. *J Virol.* 1992; 66(2):1277-81.
 101. Hall CB, Douglas RG Jr, Simons RL. Interferon production in adults with respiratory syncytial viral infection. *Ann Intern Med.* 1981; 94(1):53-5.
 102. Hall CB, Douglas RG Jr, Simons RL, Geiman JM. Interferon production in children with respiratory syncytial, influenza, and parainfluenza virus infections. *J Pediatr.* 1978; 93: 28-32.
 103. Mufson MA, Belshe RB, Örvell C, Norrby E. Subgroup characteristics of respiratory syncytial virus strains recovered from children with two consecutive infections. *J Clin Microbiol.* 1987; 25: 1535-1539.
 104. Skiadopoulos MH, Biacchesi S, Buchholz UJ, Riggs JM, Surman SR, Amaro-Carambot E, McAuliffe JM, Elkins WR, St Claire M, Collins PL, Murphy BR. The two major human respiratory metapneumovirus genetic lineages are highly related antigenically, and the fusion (F) protein is a major contributor to this antigenic relatedness. *J Virol.* 2004; 78: 6927-6937.
 105. van den Hoogen BG, Herfst S, Sprong L, Cane PA, Forleo-Neto E, de Swart RL, Osterhaus AD, Fouchier RA. Antigenic and genetic variability of human metapneumovirus. *Emerg Infect Dis.* 2004; 10:658-666.
 106. Henle G, Enders J. Mumps virus. In: Horstfall F, Tamm I, eds. *Virol and Rickettsial Infections of Man.* Philadelphia: Lippincott, 1965:755-768.
 107. Henle G, Deinhardt F, Girardi A. Cytolytic effects of mumps virus in tissue cultures of epithelial cells. *Proc Soc Exp Biol Med.* 1954; 87:386-393.
 108. Utz JP, Szwed CF, Kasel JA. Clinical and laboratory studies of mumps: II. Detection and duration of excretion of virus in urine. *Proc Soc Exp Biol Med.* 1958; 99:258-261.
 109. Arita M, Ueno Y, Masuyama Y. Complete heart block in mumps myocarditis. *Br Heart J.* 1981; 46(3):324-344.
 110. Beard CM, Benson RC, Kelalis PP, Elveback LR, Kurland LT. The incidence and

- outcome of mumps orchitis in Rochester, Minnesota, 1935 to 1947. *Mayo Clin Proc.* 1977; 52(1):3-7.
111. Philip RN, Reinhard KR, Lachman DB. Observations on a mumps epidemic in a virigin population. *Am J Hyg.* 1959; 69:91-111.
 112. Azimi PH, Cramblett HG, Haynes RE. Mumps meningoencephalitis in children. *Jama.* 1969; 207(3):509-512.
 113. Sosin DM, Cochi SL, Gunn RA, Jennings CE, Preblud SR. Changing epidemiology of mumps and its impact on university campuses. *Pediatrics.* 1989; 84(5):779-784.
 114. Glezen WP, Taber LH, Frank AL, Kasel JA. Risk of primary infection and reinfection with respiratory syncytial virus. *Am J Dis Child.* 1986; 140:543-546.
 115. Heikkinen T, Thint M, Chonmaitree T. Prevalence of various respiratory viruses in the middle ear during acute otitis media. *N Engl J Med.* 1999; 340:260-264.
 116. Sigurs N, Bjarnason R, Sigurbergsson F, Kjellman B, Björkstén B. Asthma and immunoglobulin E antibodies after respiratory syncytial virus bronchiolitis: A prospective cohort study with matched controls. *Pediatrics.* 1995; 95:500-505.
 117. Martinez FD. Role of respiratory infection in onset of asthma and chronic obstructive pulmonary disease. *Clin Exp Allergy.* 1999; 29(suppl 2):53-58.
 118. Stein RT, Sherrill D, Morgan WJ, Holberg CJ, Halonen M, Taussig LM, Wright AL, Martinez FD. Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13 years. *Lancet.* 1999; 354:541-545.
 119. Hall CB, Geiman JM, Biggar R, Kotok DI, Hogan PM, Douglas GR Jr. Respiratory syncytial virus infections within families. *N Engl J Med.* 1976; 294:414-419.
 120. Crowe JE. Human metapneumovirus as a major cause of human respiratory tract disease. *Pediatr Infect Dis J.* 2004; 23:215-221.
 121. Principi N, Bosis S, Esposito S. Human metapneumovirus in paediatric patients. *Clin Microbiol Infect.* 2006; 12:301-308.
 122. Principi N, Esposito S, Bosis S. Human metapneumovirus and lower respiratory tract disease in children. *N Engl J Med.* 2004; 350:1788-1790.
 123. Anderson RM, Crombie JA, Grenfell BT. The epidemiology of mumps in the UK: A preliminary study of virus transmission, herd immunity and the potential impact of immunization. *Epidemiol Infect.* 1987; 99:65-84.
 124. Tecle T, Bottiger B, Örvell C, Johansson B. Characterization of two decades of temporal co-circulation of four mumps virus genotypes in Denmark: identification of a new genotype. *J Gen Virol.* 2001; 82 (pt11):2675-2680.

125. Teele T, Johansson B, Jejcic A, Forsgren M, Örvell C. Characterization of three co-circulation genotypes of the small hydrophobic protein gene of mumps virus. *J Gen Virol.* 1998; 79(pt 12):2929-2937.
126. Örvell C, Kalantari M, Johansson B. Characterization of five conserved genotypes of the mumps virus small hydrophobic (SH) protein gene. *J Gen Virol.* 1997; 78(pt1):91-95.
127. Kim SH, Song KJ, Shin YK, Kim JH, Choi SM, Park KS, Baek LJ, Lee YJ, Song JW. Phylogenetic analysis of the small hydrophobic (SH) gene of mumps virus in Korea: identification of a new genotype. *Microbiol Immunol.* 2000; 44(3):173-177.
128. Kunkel U, Schreier E, Siegl G, Schultze D. Molecular characterization of mumps virus strains circulating during an epidemic in eastern Switzerland 1992/93. *Arch Virol.* 1994; 136(3-4):433-438.
129. Yeo RP, Afzal MA, Forsey T, Rima BK. Identification of a new mumps virus lineage by nucleotide sequence analysis of the SH gene of ten different strains. *Arch Virol.* 1993; 128(3-4):371-377.
130. Uchida K, Shinohara M, Shimada SJ, Segawa Y, Kimura K, Hoshino Y. Characterization of the F gene of contemporary mumps virus strains isolated in Japan. *Microbiol Immunol.* 2003; 47:167-172.
131. Saito H, Takahashi Y, Harata S, Tanaka K, Sato H, Suto T, Yamada A, Yamazaki S, Morita M. Cloning and Characterization of the genomic RNA sequence of the mumps virus strain associated with a high incidence of aseptic meningitis. *Microbiol Immunol.* 1998; 42(2):133-137.
132. Afzal MA, Buchanan J, Heath AB, Minor PD. Clustering of mumps virus isolates by SH gene sequence only partially reflects geographical origin. *Arch Virol.* 1997; 142(2):227-238.
133. Mufson MA, Levine HD, Wasil RE, Mocega-Gonzalez HE, Krause HE. Epidemiology of respiratory syncytial virus infection among infants and children in Chicago. *AM J Epidemiol.* 1973; 98:88-95.
134. Winter GF, Inglis JM. Respiratory viruses in children admitted to hospital in Edinburgh 1972-1985. *J Infect.* 1987; 15:103-107.
135. Brandt CD, Kim HW, Arrobbio JO, Jeffries BC, Wood SC, Chanock RM, Parrott RH. Epidemiology of respiratory syncytial virus infection in Washington, DC. 3. Composite analysis of eleven consecutive yearly epidemics. *Am J Epidemiol.* 1973; 98:355-364.
136. Cane PA, Weber M, Sanneh M, Dackour R, Pringle CR, Whittle H. Molecular of respiratory syncytial virus in the Gambia. *Epidemiol Infect.* 1999; 122:155-160.

137. Mufson MA, Belshe RB, Örvell C, Norrby E. Respiratory syncytial virus epidemics: variable dominance of subgroup A and B strain among children, 1981-1986. *J Infect Dis.* 1988; 157:143-148.
138. Peret TC, Hall CB, Schnabel KC, Golub JA, Anderson LJ. Circulation patterns of genetically distinct group A and B strains of human respiratory syncytial virus in a community. *J Gen Virol.* 1998; 79:2221-2229.
139. Waris M. Pattern of respiratory syncytial virus epidemics in Finland: two-year cycles with alternating prevalence of group A and B. *J Infect Dis.* 1991;163:464-469.
140. Graman PS, Hall CB. Epidemiology and control of nosocomial viral infections. *Infect Dis Clin North Am.* 1989; 3:815-841.
141. Gerna G, Campanini G, Rovida F, Sarasini A, Lilleri D, Paolucci S, Marchi A, Balanti F, Revello MG. Changing circulation rate of human metapneumovirus strains and types among hospitalized pediatric patients during three consecutive winter-spring seasons. *Arch Virol.* 2005; 150: 2365-2375.
142. Meurman O, Vainionpää R, Rossi T, Hänninen P. Viral etiology of parotitis. *Scand J Infect Dis.* 1983; 15(2):145-148.
143. Grillner L, Blomberg J. Hemolysis-in-gel and neutralization tests for determination of antibodies to mumps virus. *J Clin Microbiol.* 1976; 4(1):11-15.
144. Yoshida N, Fujino M, Miyata A, Nagai T, Kamada M, Sakiyama H, Ihara T, Kumagai T, Okafuji T, Nakayama T. Mumps virus reinfection is not a rare event confirmed by reverse transcription loop-mediated isothermal amplification. *J Med Virol.* 2008;80:517-523.
145. Bitsko RH, Cortese MM, Dayan GH, Rota PA, Lowe L, Iversen SC, Bellini WJ. Detection of RNA of mumps virus during an outbreak in a population with a high level of measles, mumps, and rubella vaccine coverage. *J Clin Microbiol.* 2008;46:1101-1103.
146. Meurman O, Hänninen P, Krishna RV, Ziegler T. Determination of IgG- and IgM-class antibodies to mumps virus by solid-phase enzyme immunoassay. *J Virol Methods.* 1982; 4(4-5):249-256.
147. Gut JP, Lablache C, Behr S, Kim A. Symptomatic mumps virus reinfections. *J Med Virol.* 1995; 8(2):17-23.
148. Lennette DA, Lennette EH. Rapid diagnosis of mumps virus infections by immunofluorescence methods. *J Clin Microbiol.* 1975; 2:81-84.
149. Forsey T, Mawn JA, Yates PJ, Bentley ML, Minor PD. Differentiation of vaccine and

- wild mumps viruses using the polymerase chain reaction and dideoxynucleotide sequencing. *J Gen Virol*. 1990; 71(pt 4):987-990.
150. Deibel R, Schryver GD. Viral antibody in the cerebrospinal fluid of patients with acute central nervous system infections. *J Clin Microbiol*. 1976; 3(4):397-401.
 151. Boddicker JD, Rota PA, Kreman T, Wangeman A, Lowe L, Hummel KB, Thompson R, Bellini WJ, Pentella M, Desjardin LE. Real-time reverse transcription-PCR assay for detection of mumps virus RNA in clinical specimens. *J Clin Microbiol*. 2007; 45:2902-2908.
 152. Cannon MJ. Microplaque immunoperoxidase detection of infectious respiratory syncytial virus in the lungs of infected mice. *J Virol Methods*. 1987; 16:293-301.
 153. Trepanier P, Payment P, Trudel M. A simple and rapid microassay for the titration of human respiratory syncytial virus. *J Virol Methods*. 1980; 1:343-347.
 154. Waris M, Meurman O, Mufson MA, Ruuskanen O, Halonen P. Shedding of infectious virus and virus antigen during acute infection with respiratory syncytial virus. *J Med Virol*. 1992; 38:111-116.
 155. Tang YW, Heimgartner PJ, Tollefson SJ, Berg TJ, Rys PN, Li H, Smith TF, Persing DH, Wright PF. A colorimetric microtiter plate PCR system detects respiratory syncytial virus in nasal aspirates and discriminates subtypes A and B. *Diagn Microbiol Infect Dis*. 1999; 4:333-337.
 156. Richardson LS, Yolken RH, Belshe RB, Camargo E, Kim HW, Chanock RM. Enzyme-linked immunosorbent assay for measurement of serological response to respiratory syncytial virus infection. *Infect Immun*. 1978; 0:660-664.
 157. Langedijk JP, Brandenburg AH, Middel WG, Osterhaus A, Melen RH, Van Oirschot JT. A subtype-specific peptide-based enzyme immunoassay for detection of antibodies to the G protein of human respiratory syncytial virus is more sensitive than routine serological tests. *J Clin Microbiol*. 1997; 35:1656-1660.
 158. Mackay IM, Jacob KC, Woolhouse D, Waller K, Symmis MW, Whitley DM, Siebert DJ, Nissen M, Sloots TP. Molecular assay for the detection of human metapneumovirus. *J Clin Microbiol*. 2003; 41:100-105.
 159. Hamelin M, Boivin G. Development assay for human metapneumovirus serology based on a viral protein. *Clin Diagn Lab Immunol*. 2005; 12:249-253.
 160. Leung J, Esper F, Weibel C, Kahn JS. Seroepidemiology of human metapneumovirus

- (hMPV) on the basis of a novel enzyme-linked immunosorbent assay utilizing hMPV fusion protein expressed in recombinant vesicular stomatitis virus. *J Clin Microbiol.* 2005; 43:1213-1219.
- 161.Ebihara T, Endo R, Ma X, Ishiguro N, Kikuta H. Detection of human metapneumovirus antigens in nasopharyngeal secretions by an immunofluorescent-antibody test. *J Clin Microbiol.* 2005; 43:1138-1141.
 - 162.Landry ML, Ferguson D, Cohen S, Peret TC, Eedman DD. Detection of human metapneumovirus in clinical samples by immunofluorescence staining of shell vial centrifugation cultures prepared from three different cell lines. *J Clin Microbiol.* 2005; 43:1950-1952.
 - 163.Gellis SS, McGuinness AC, Peters M. A study of the prevention of mumps orchitis by gammaglobulin. *Am J Med Sci.* 1945; 210:661-664.
 - 164.Casella R, Leibundgut B, Lehmann K, Gasser TC. Mumps orchitis: Report of a mini-epidemic. *J Urol.* 1997; 158:2158-2161.
 - 165.Jeng MJ, Lemen RJ. Respiratory syncytial virus bronchiolitis. *Am Fam Physician.* 1997; 55:1139-1146, 1149-1150.
 - 166.Kimpen JL, Schaad UB. Treatment of respiratory syncytial virus bronchiolitis:1995 poll of members of the European Society for Paediatric Infectious Diseases. *Pediatr Infect Dis J.* 1997; 16:479-481.
 - 167.McCarthy CA, Hall CB. Recent approaches to the management and prevention of respiratory syncytial virus infection. *Curr Clin Top Infect Dis.* 1998; 18:1-18.
 - 168.Anderson LJ, Parker RA, Strikas RL. Association between respiratory syncytial virus outbreaks and lower respiratory tract deaths of infants and young children. *J Infect Dis.* 1990; 161(4):640-646.
 - 169.Rodriguez WJ. Management strategies for respiratory syncytial virus infections in infants. *J Pediatr.* 1999; 135:45-50. Review.
 - 170.Randolph AG, Wang EE. Ribavirin for respiratory syncytial virus lower respiratory tract infection. A systematic overview. *Arch Pediatr Adolesc Med.* 1996; 150(9):942-947.
 - 171.Prince GA, Hemming VG, Horswood RL, Chanock RM. Immunoprophylaxis and immunotherapy of respiratory syncytial virus infection in the cotton rat. *Virus Res.* 1985; 3(3):193-206.
 - 172.Prince GA, Hemming VG, Horswood RL, Baron PA, Chanock RM. Effectiveness of topically administered neutralizing antibodies in experimental immunotherapy of respiratory syncytial virus infection in cotton rats. *J Virol.* 1987; 61(6):1851-1854.

173. Mally R, DeVincenzo J, Ramilo O, Dennehy PH, Meissner HC, Gruber WC, Sanchez PJ, Jafri H, Balsley J, Carlin D, Buckingham S, Vernacchio L, Ambrosino DM. Reduction of respiratory syncytial virus (RSV) in tracheal aspirates in intubated infants by use of humanized monoclonal antibody to RSV F protein. *Infect Dis.* 1998; 178(6):1555-61.
174. Rodriguez WJ, Gruber WC, Welliver RC, Groothuis JR, Simoes EA, Meissner HC, Hemming VG, Hall CB, Lepow ML, Rosas AJ, Robertsen C, Kramer AA. Respiratory syncytial virus (RSV) immune globulin intravenous therapy for RSV lower respiratory tract infection in infants and young children at high risk for severe RSV infections: Respiratory Syncytial Virus Immune Globulin Study Group. *Pediatrics.* 1997; 99(3):454-461.
175. Rimensberger PC, Burek-Kozłowska A, Morell A, Germann D, Eigenmann AK, Steiner F, Burger R, Kuenzli M, Schaad UB. Aerosolized immunoglobulin treatment of respiratory syncytial virus infection in infants. *Pediatr Infect Dis J.* 1996; 15(3):209-216.
176. Wyde PR, Chetty SN, Jewell AM, Boivin G, Piedra PA. Comparison of the inhibition of human metapneumovirus and respiratory syncytial virus by ribavirin and immune serum globulin in vitro. *Antiviral Res.* 2003; 60(1):51-59.
177. Hamelin ME, Abed Y, Boivin G. Human metapneumovirus: a new player among respiratory viruses. *Clin Infect Dis.* 2004; 38:983-990.
178. Tecle T, Mickiene A, Johansson B, Lindquist L, Örvell C. Molecular characterisation of two mumps virus genotypes circulating during an epidemic in Lithuania from 1998 to 2000. *Arc Virol.* 2002;147:243:253.
179. Örvell, C., Norrby, E. and Mufson, M.A. Preparation and characterization of monoclonal antibodies directed against five structural components of human respiratory syncytial virus subgroup B. *J. Gen. Virol.* 1987; 68:3125–3135.
180. Allander T, Tammi MT, Eriksson M, Bjerkne A, Tiveljung-Lindell A, Andersson B. Cloning of a human parvovirus by molecular screening of respiratory tract samples. *Proc Natl Acad Sci U S A.* 2005; 102:12891-12896.
181. van de Peer Y and De Wachter, R. TREECON for Windows: a software package for the construction and drawing of evolutionary trees for the MICROSOFT Windows Environment. *Appl. Biosci.* 1994; 10:569–570.
182. Rotzén Östlund M, Tiveljung Lindell A, Stenler S, Riedel H, Zwegyberg Wirgart B, Grillner L. Molecular epidemiology and genetic variability of respiratory syncytial virus (RSV) in Stockholm, 2002-2003. *J Med Virol.* 2008;80:159-167.
183. Rafiefard F, Johansson B, Tecle T, Örvell C. Molecular epidemiology of respiratory

- syncytial virus (RSV) of group A in Stockholm, Sweden, between 1965 and 2003. *Virus Res.* 2004; 105:137-145.
184. Maggi F, Pifferi M, Vatteroni M, Fornai C, Tempestini E, Anzilotti S, Lanini L, Andreoli E, Ragazzo V, Pistello M, Specter S, Bendinelli M. Human metapneumovirus associated with respiratory tract infections in a 3-year study of nasal swabs from infants in Italy. *J Clin Microbiol.* 2003; 41:2987-2991.
 185. Weigl JA, Puppe W, Meyer CU, Berner R, Forster J, Schmitt H J, Zepp F. Ten years' experience with year-round active surveillance of up to 19 respiratory pathogens in children. *Eur J Pediatr.* 2007; 166:957-966.
 186. Boivin G, Mackay I, Sloots T P, Madhi S, Freymuth F, Wolf D, Shemer-Avni Y, Ludewick H, Gray GC, LeBlanc E. Global genetic diversity of human metapneumovirus fusion gene. *Emerg Infect Dis.* 2004; 10:1154-1157.
 187. Mahalingam S, Schwarze J, Zaid A, Nissen M, Sloots T, Tauro S, Storer J, Alvarez R, Tripp RA. Perspective on the host response to human metapneumovirus infection: what can we learn from respiratory syncytial infections? *Microbes Infect.* 2006; 8:285-293.
 188. van den Hoogen BG, van Doornum GJ, Fockens JC, Cornelissen JJ, Beyer WE, de Groot R, Osterhaus AD, Fouchier RA. Prevalence and clinical symptoms of human metapneumovirus infection in hospitalized patients. *J Infect Dis.* 2003; 188:1571-1577.
 189. Dollner H, Risnes K, Radtke A, Nordbo SA. Outbreak of human metapneumovirus infections in Norwegian children. *Pediatr Infect Dis J.* 2004; 23:436-440.

12 APPENDIX

PAPER I: Characterization of mumps virus strains with varying neurovirulence.

PAPER II: Molecular epidemiology of respiratory syncytial virus (RSV) of group A in Stockholm, Sweden, between 1965 and 2003.

PAPER III: Genotyping of respiratory syncytial virus (RSV) group A in Stockholm, Sweden, using PCR and two-dimensional melting curve analysis.

PAPER IV: Epidemiologic characteristics and seasonal distribution of human metapneumovirus in five epidemic seasons in Stockholm, Sweden 2002-2006.