RESPIRATORY ILLNESS AND INFECTIONS WITH HUMAN METAPNEUMOVIRUS AND RESPIRATORY SYNCYTIAL VIRUS IN YOUNG CHILDREN

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II. von Linstow ML, Eugen-Olsen J, Koch A, Winther TN, Westh H, Høgh B. Excretion patterns of human metapneumovirus and respiratory syncytial virus among young children. Eur J Med Res 2006;11:329-35.

III. von Linstow ML, Holst KK, Larsen K, Koch A, Andersen PK, Høgh B. Acute respiratory symptoms and general illness during the first year of life: A population-based birth cohort study. Submitted.

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Abbreviations

ARTI	Acute respiratory tract infection
RSV	Respiratory syncytial virus
LRTI	Lower respiratory tract infection
hMPV	Human metapneumovirus
APV	Avian pneumovirus
URTI	Upper respiratory tract infection
n-CPAP	Nasal-continuous positive airway pressure
(RT)-PCR	(Reverse transcription)-polymerase chain reaction
IFA	Immunofluorescence assay
EIA	Enzyme immonoassays
GA	Gestational age
NPA	Nasopharyngeal aspirate
PBS	Phosphate-buffered saline
OD	Optical density
RT	Room temperature
Ct	Threshold cycle
nd	Not done
na	Not available
S.T.A.R	Stool transportation and recovery
FRET	Fluorescence resonance energy transfer
PDV	Phocine Distemper Virus
ELISA	Enzyme-linked immunosorbent assay
M-PBS	PBS with 2% skim-milk powder
HRP	Horseradish peroxidase
TMB	Tetramethyl benzidine
SD	Standard deviation
PAU	Pharmacia Arbitrary Units
OR	Odds ratio
RR	Relative risk

IQR	Inter quartile ranges
CI	Confidence interval
GEE	Generalised estimating equations
SPT	Skin prick test

Contents

1. Introduction

Acute respiratory tract infection (ARTI) in infants and young children is a significant public health problem worldwide. A substantial part of respiratory tract infections is associated with viruses, and although rarely fatal in industrialised countries, they are a source of significant morbidity and carry a considerable economic burden.

Respiratory syncytial virus (RSV) is the leading cause of lower respiratory tract infection (LRTI) in young children[17]. Only about 40% of children suspected to have an RSV infection have a positive test result and in many cases – up to 50% - the etiological agent remains unknown[18], which is in part due to diagnostic limitations. A proportion of ARTIs may be caused by still unknown pathogens. In 2001, a hitherto unknown virus was isolated from respiratory specimens from children with ARTI in the Netherlands, and the virus was named human metapneumovirus (hMPV)[136]. The discovery of hMPV forms the basis of the studies in the present thesis. As the epidemiology and clinical impact of RSV infection are well described, infections due to hMPV and RSV were compared in the studies.

2. Background

3.1. Illness in infancy

The most common illness in infancy and childhood is ARTI. The epidemiology of ARTI in infants differs from that of older children and adults. Early childhood is a time of particular susceptibility to respiratory ailments, and infants often develop more severe symptoms when infected with a respiratory virus. This is mainly due to immune immaturity and the smaller anatomy of the lower airways in infants. Most studies report incidences of 4-7 respiratory episodes per year, depending on the design of the study, demographics of the populations studied, definitions of respiratory illnesses, and the methods of surveillance employed[25, 74, 81, 85, 96]. Several risk factors for severe LRTI in developed countries have been described, such as daycare attendance[67, 68, 78, 132] and lack of

breastfeeding[19]. In Denmark, this has led to recommendations of a longer maternity leave and an aggressive breastfeeding policy. Other risk factors such as crowding and siblings, passive smoking, low socioeconomic status, psychosocial factors, male gender, and low birth weight are in many studies also found to be associated with lower respiratory tract disease, while other studies do not report such associations[43].

Risk factors for upper respiratory tract infections (URTI), which are even more prevalent among children and have substantial impact on the disease burden experienced by families, have only been sparsely investigated[68, 85]. Likewise, not much is known about minor general illness in infancy as parents deal with most of these symptoms without seeking medical advice[59].

3.2. Respiratory viruses

A large number of viral pathogens belonging to different virus families have been associated with ARTI in humans; RSV, hMPV, parainfluenza virus, influenza virus, adenovirus, coronavirus OC43 and 229E, rhinovirus and enterovirus. Since the discovery of hMPV several new viruses have been identified as a cause of ARTI in humans; SARS-CoV, coronaviruses HKU1 and NL63, human bocavirus and several parechoviruses. In the following sections only RSV and hMPV, both members of the family *Paramyxoviridae*, will be described in detail.

3.3. Paramyxoviridae family

Paramyxoviruses are enveloped, cytoplasmic viruses containing non-segmented, single-stranded, negative-sense RNA. The *Paramyxoviridae* family consists of several major pathogens and is divided into two subfamilies, the *Paramyxovirinae* and the *Pneumovirinae* (Figure 1). The *Pneumovirinae* subfamily has two genera: the pneumoviruses and the metapneumoviruses. The classification of the two genera is based primarily on their gene constellation: metapneumoviruses lack two non-structural proteins, and the gene order is different from that of pneumoviruses. Human RSV is the type species of the genus *Pneumovirus* and was until the discovery of hMPV the only human pathogen identified in the subfamily *Pneumovirinae.* Avian pneumovirus (APV) causes URTI in turkeys and other birds and was the sole member of the *Metapneumovirus* genus until 2001.

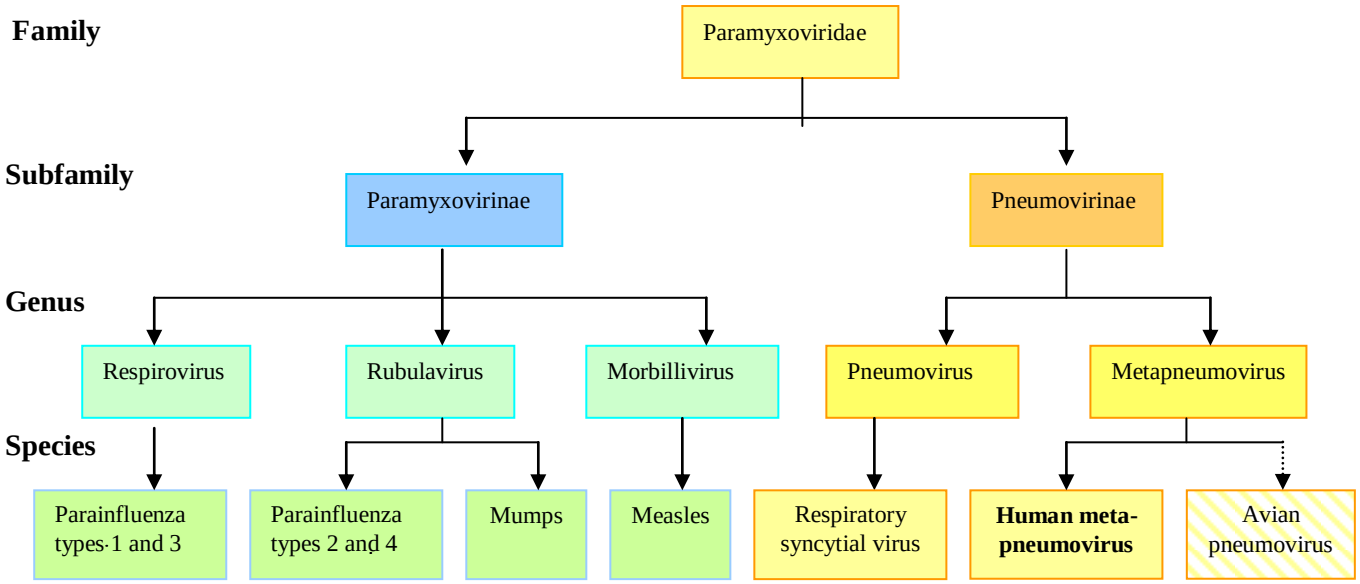

Figure 1. Classification of viral pathogens of the *Paramyxoviridae* family that infect humans and avian pneumovirus (APV) which causes upper respiratory tract illness in turkeys. APV is the pathogen most closely related to human metapneumovirus (hMPV). Respiratory syncytial virus is the human pathogen closest related to hMPV.

3.4. Respiratory syncytial virus

3.4.1. Epidemiology and clinical manifestations

Respiratory syncytial virus was first isolated in 1956 from a chimpanzee, and its infectivity in humans was documented the next year[14]. RSV has a worldwide distribution and is one of the main causes of ARTI. In temperate climates, RSV produces yearly outbreaks from winter to early spring[94]. Two subgroups of RSV exist and both strains circulate concurrently[52]. Approximately 2/3 of all children acquire RSV infection during their first year of life; virtually all children have been infected by 24 months of age, and about half have experienced two infections[42]. Primary RSV infection mostly causes a febrile URTI and is commonly associated with acute otitis media[10, 106], but up to one third of primary infections also involve the lower airways[42]. In young infants, the clinical manifestations may be non-specific, presenting with poor feeding, irritability, lethargy, or apnoea[128]. In addition to respiratory symptoms, severe RSV infection has been associated with extrapulmonary symptoms, and RSV or its genetic material have been isolated from cerebrospinal fluid, myocardium, liver, and peripheral blood indicating a systemic spread at least in some individuals[30].

A study from east Denmark found that 3.4% of RSV-infected children below the age of 6 months required hospitalisation during the winter season 1995-1996[72]. A recent study from the US reports the annual hospitalisation rate to be around 2.5%[80]. In affluent countries, very few previously healthy children suffer life-threatening infections, and deaths are practically confined to those who are immunocompromised or who have preexisting cardiorespiratory disease[94]. However, due to the common occurrence of RSV infection, even a low mortality rate may have a marked impact on the total mortality of young children.

3.4.2. Transmission

RSV is highly transmissible: exposure to an RSV-infected infant over 2-4 hours results in infection among 71% of the exposed individuals[46], and in a family study of RSV-infected infants, 46% of the exposed family members became infected[49]. Close contact appears to be necessary for infection to spread from one person to another; in one study, no one sitting at a distance greater than 1.8 m from RSV-infected infants became infected[46]. Spread occurs through large droplets of respiratory secretions or through direct contact with contaminated surfaces where live virus can survive for up to six hours[48]. RSV is secreted in nasopharyngeal secretions usually for 4-7 days[45, 49]. However, some infants may shed virus for up to four weeks and immunocompromised individuals for many months, thus increasing its contagious nature[47]. Older children and adults usually shed virus for 2-4 days[51].

3.4.3. Pathogenesis and Immunity

The incubation period of RSV-induced respiratory disease is 3-5 days[126]. Inoculation of upper respiratory tract epithelial cells occurs primarily through the eyes or nose with subsequent cell-to-cell transfer of the virus to the lower respiratory tract where it may produce bronchiolitis and/or pneumonia[21].

Immunity to RSV following natural infection is transient and imperfect, and re-infections are seen throughout life[51, 57]. Both serum and secretory antibodies play a role in the protection against RSV infection. Resistance to RSV infection in the upper respiratory tract is most likely mediated by local secretory IgA, whereas serum IgG neutralising antibodies provide protection in the lower respiratory tract[126]. Young infants mount poor antibody responses to primary infection, which may be secondary to the relative immunologic immaturity of the infants and/or to a suppressive effect of

maternally transmitted transplacental antibodies[98]. Although complete protection against infection may not exist, RSV-specific immunity can protect against severe LRTI. Furthermore, cellular immune responses are thought to play an important role in clearing RSV.

3.4.4. Risk of severe disease

High-risk groups for severe RSV disease are: children born prematurely[15, 99]; children with concurrent heart or lung disease[37, 99]; immunocompromised children[50]; age<6 weeks[99]; and the elderly[35]. In addition, crowding in the home, siblings, daycare attendance, passive smoking, low birth weight, month of birth, male sex, and a family history of asthma and atopy have been associated with severe RSV disease[15, 55, 58, 77, 103, 116, 134]. Some, but not all studies, find a protective effect of maternal antibody level and breastfeeding[58, 112]. To our knowledge, no studies have investigated risk factors for RSV-associated upper respiratory illness.

3.4.5. RSV and atopic disease

Whether RSV infection at a young age is causal in the development of atopic disease like asthma and allergy remains a subject of debate. In most studies, the prevalence of asthma, wheezing, and IgE sensitisation after RSV bronchiolitis has been higher in the index than the control group[119, 123-125]. Some studies find that this association declines over time[71, 111, 129], and according to a Finnish study early RSV hospitalisation results in reduction of skin prick test positivity 6-10 years after hospitalisation[64]. The potential role of mild RSV infections in infancy in the development of atopic disease at a later age is even more unclear. Forster *et al.* have shown that mild RSV infections at a young age can promote aeroallergen sensitisation during the first year of life, but they were unable to show atopic manifestations during the first two years of life[38]. Finally, it could also be the other way around; that atopic disposition, wheezing and atopic disorders are determinants for RSV hospitalisation[131].

3.4.6. Prevention and treatment

Despite many efforts, at present no safe and effective vaccine against RSV infection is available. A humanised monoclonal antibody (palivizumab) has been approved for its use in high-risk infants to protect against serious disease due to RSV, though it does not prevent infection. Thorough handwashing is the most effective method of interrupting transmission of RSV in the homes, in daycare institutions, and in hospital environments[12, 62, 117, 118].

Treatment of RSV bronchiolitis is supportive with supplemental oxygen therapy or nasal-continuous positive airway pressure (n-CPAP), suction of secretions from the airways, and sufficient fluid therapy. Antibiotics, corticosteroids, bronchodilator drugs, ribavirin, and physiotherapy are not recommended routinously[44].

3.5. Human metapneumovirus

3.5.1. Discovery of an unknown virus

In 2001, Dutch researchers isolated an agent from respiratory specimens collected during a 20-year period from 28 young children with ARTI[136]. The agent induced cytopathic effects on cultured cells, and electron microscopy revealed viral-like particles, but immunological assays using virus-specific antibodies and polymerase chain reaction (PCR)-based methods using specific primers for known viral pathogens failed to identify this agent. Experimentally infected monkeys developed URTI and the virus could be recovered from infected animals. Ultimately, genome sequences of this novel pathogen were obtained by using a molecular biology technique known as randomly arbitrarily primed PCR. On the basis of morphological, biochemical, and genetic features, hMPV was found to be closely related to APV and was tentatively classified in the *Metapneumovirus* genus of the *Paramyxoviridae* family. Phylogenetic analysis revealed the existence of 2 genotypes of hMPV. Screening of banked human sera showed that hMPV had been circulating for at least 50 years, suggesting that the virus did not recently "jump" to the human population from an animal reservoir such as birds[136].

3.5.2. Epidemiology and clinical manifestations

Since its discovery in 2001, hMPV has been identified as a cause of respiratory tract illness in all age groups worldwide. In most studies, hMPV accounts for 4-8% of the ARTI cases in hospitalised children[6, 9, 31, 32, 39, 93, 97, 138] but frequencies up to 21% have been reported[24]. In temperate climate zones, hMPV circulates primarily during late winter/early spring overlapping or following the peak of RSV activity[2, 40, 105, 113, 151]. The clinical symptoms associated with hMPV infections are best studied in hospitalised children and are very similar to those induced by RSV[6, 138, 143, 148]. Most frequent are cough, fever, coryza, and wheezing, but also symptoms such as diarrhoea, vomiting, rash, febrile convulsions, conjunctivitis and otitis media have been associated with hMPV infection[6, 39, 107, 127, 137, 149]. Very severe symptoms have been noted in children infected with both hMPV and RSV in some studies[70, 121] but not in others[79, 140, 151]. hMPV can cause an influenza-like

illness in adults and has been associated with wheezing and asthma in young children[31, 32, 63, 92, 107, 138, 149]. hMPV is uncommon in asymptomatic children and adults[33, 136, 149]. Children with underlying illnesses such as a history of prematurity, congenital heart or lung disease, or immunodeficiency may be predisposed to severe hMPV disease[65]. However, risk factors for severe or early hMPV infections have not been thoroughly investigated. Furthermore, information on hMPV infection in non-hospitalised children is very sparse.

3.5.3. Pathogenesis, transmission and immunity

Animal experiments have shown that hMPV replicates in the respiratory epithelium only[3, 73], and the current understanding is that during infection both the virus and the disease are limited to the respiratory tract. However, findings from different groups indicate that hMPV infection is associated with encephalitis and may thus cause disseminated infection in some individuals[41, 66, 120]. A recent study suggests that the pathogenesis of hMPV-associated LRTI involves co-infection with pneumococcus, as vaccination with pneumococcal conjugate vaccine reduces the incidence of hMPV (as well as other respiratory viruses) LRTI hospitalisations in young children[88, 89]. The incubation period of hMPV is unknown but has been estimated to be 4-6 days[27]. hMPV infection in children appears to have a great impact on their families; in one study 12.5% of the family members of hMPV-positive children had a disease similar to that of the infected child, which was significantly more than for RSV-positive children[8]. The shedding period and the modes of transmission have not yet been investigated, although they are considered similar to those of RSV. More than 90% seroprevalence has been found by the age of five[28, 83, 136], but like other respiratory viruses, hMPV infection does not confer lasting immunity and re-infections are seen throughout life.

3.6. Laboratory diagnosis of RSV and hMPV

Virus isolation from respiratory specimens by cell culture has long been the gold standard for diagnosis of viral infection. However, cell culture requires specimens to be transported and stored under ideal conditions, and it takes several days before a diagnosis can be established. hMPV grows poorly in cell culture, replicates in few cell lines only, and shows late cytopathic effect, which are some of the reasons for its late identification.

In recent years, reverse transcriptase-polymerase chain reaction (RT-PCR) has proved to be a highly sensitive and specific method for the diagnosis of RSV and hMPV infections[1, 34]. Especially real-

time RT-PCR assays are rapid, specific, quantitative, and highly sensitive and are capable of subtyping RSV in clinical specimens[75, 76, 90, 95]. Other methods used for antigen detection are immunofluorescence assays (IFA) and enzyme immonoassays (EIA), using virus-specific antibodies. These methods are rapid, but not as sensitive as RT-PCR[1, 13, 29, 108].
Serological tests detecting virus-specific antibodies (ELISA, IFA, neutralization and haemagglutination inhibition tests) provide immunologic evidence of infection after the acute phase of the disease and are only useful for epidemiological studies or retrospective diagnosis.

3. Aims

The overall aim of this thesis was to study the clinical epidemiology of hMPV compared with RSV in hospitalised and non-hospitalised children in the area of Copenhagen.

The specific aims were

1. To determine the frequencies and clinical features of hMPV and RSV infections in children hospitalised with acute respiratory tract infection (Paper I).
2. To examine the excretion patterns of hMPV and RSV in children (Paper II).
3. To measure the incidence and prevalence of acute respiratory symptoms and overall morbidity in unselected infants in the community and to identify risk factors for respiratory symptoms in the first year of life (Paper III).
4. To study the clinical symptoms and risk factors associated with hMPV and RSV infections in unselected infants in the community during the first year of life (Paper IV).

4. Methods

4.1. The study area

The studies in the present thesis involve children residing in the area of Hvidovre Hospital, Denmark, which serves an area of Copenhagen with 396,000 persons (35% of the total population of Greater Copenhagen) and has around 5,500 births a year. The first study also includes children residing in the area of Amager Hospital, which is smaller and serves approximately 158,000 citizens. Both hospitals are located in a city area near the capital with housing of both flats and free houses, but no country-area. In this area, approximately 14% of 20-45-year-old inhabitants have a high academic degree and 12% have a medium-long education. In families with children, 52% live with one child and in 12% of families there are three or more children[20]. In Denmark, 27% of the adult population smoke[133]. The climate is temperate.

4.2. Study populations

The studies forming the basis of the present thesis were carried out in three different groups of children:

1. *Prevalence study:* The first study included 383 stored routine nasopharyngeal aspirates (NPA) obtained from 374 children hospitalised with ARTI at the Departments of Paediatrics, Hvidovre or Amager Hospital, during the RSV seasons (November-May) 1999-2000 and 2001-2002. The NPAs were analysed for the presence of hMPV and RSV by RT-PCR and the medical records of all children were systematically reviewed.

2. *Excretion study:* The second study included children admitted to the Department of Paediatrics, Hvidovre Hospital, during the winter season 2003-2004 and who were tested positive for hMPV or RSV in a routine NPA. The children were followed for three weeks with weekly home visits to examine the excretion of viral RNA in different secretions; at inclusion and each week, one NPA specimen, one saliva sample, one urine sample and one stool sample were taken from each child. In addition, sweat and blood samples were obtained at inclusion.

3. *Birth cohort study:* In this study, children were recruited from the post-natal ward at Hvidovre Hospital. Due to logistic restraints and in accordance with statistical power calculations approximately 250 healthy newborns were enrolled into the study during a 12-month period from May 2004 to May 2005. To ensure that children were sampled equally throughout the year approximately 20 children, of whom half had siblings, were enrolled each month on predesignated weeks. The inclusion criteria for participation in the study were: Infants free of obvious health problems, and for practical purposes living within 11 km of Hvidovre Hospital. Exclusion criteria were: Infants whose parents did not understand or speak Danish or English; infants whose mothers had a serious psychiatric disorder; infants with congenital diseases; and if change of address to outside the area of Hvidovre Hospital was planned within 12 months of enrolment. All children were followed for 12 months with monthly home visits. At every home visit children had a nasal swab taken. Figure 2 outlines the study design of the birth cohort study.

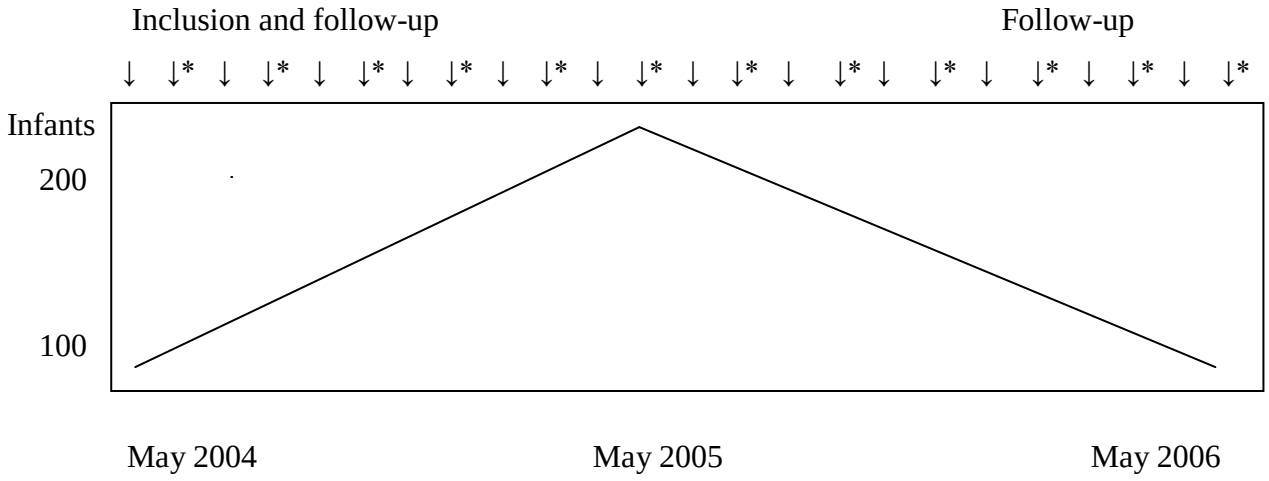

Figure 2. Study design of birth cohort study.

↓ = Monthly visits (health diary, nasal swab)

* = Every second month (interview)

Blood sample at 5 days and at 12 months of age

4.3. Clinical data

The medical records of children included in studies 1 and 2 were systematically reviewed and information was registered on demographic characteristics, symptoms during hospitalisation, physical examination, treatment, and final diagnosis. In the excretion study, parents were interviewed weekly about their child's present and previous illnesses and related diseases in household members. In addition, information was obtained on gestational age (GA), duration of breastfeeding, daycare attendance, smoking in the household, and atopic dispositions.

In the birth cohort study, parents were provided monthly with a health diary displaying 12 different symptoms and clinical signs, doctor's visits, hospital admissions and medicine (see Appendix I). At the first home visit the parents were interviewed about household members, parents' education and employment, ethnicity, birth weight of the included infant, breastfeeding, dispositions (hay fever, asthma and atopic dermatitis) and exposures (smoking in homes, smoking during pregnancy, pets, moist, carpets, drying clothes inside). Questions concerning factors that could change over time were repeated every second month. Ethnicity was defined as Western/non-Western according to the biologic parents' place of birth. Only if both parents were born in a Western country, ethnicity was defined as Western. Socioeconomic status was classified according to the Danish social classification system choosing the social group, which comes first in the order 1 to 5 in families where the mother and father were classified in two different categories[54]. Allergic dispositions were regarded as present if the parents answered "yes" to the question "has anyone in the family ever had asthma, hay fever or atopic dermatitis?" Dispositions by first- and second-degree relatives were included in the analyses. Children were seen in the hospital for the final interview and blood sampling during the month they turned one year or the following month.

4.4. Definitions of illness episodes

4.4.1. Definitions used in Paper I:

- *ARTI:* A period with symptoms and clinical signs of URTI or LRTI.
- *URTI:* One or more of the following signs present: nasal discharge, cough, a bulging red tympanic membrane and pharyngo-tonsillar erythema or exudate without signs of LRTI.
- *LRTI:* Signs of URTI together with chest indrawing and tachypnoea combined with rhonchi and/or crepitation. All children who required respiratory support were included in the LRTI group.

- *Asthmatic bronchitis:* Symptoms of ARTI in combination with wheezing and/or rhonchi on lung auscultation.
- *New ARTI episode*: An episode of ARTI with a 7-day interval free of symptoms to the previous ARTI episode.

4.4.2. Definitions used in Paper III:

- *ARTI:* Nasal discharge together with one or more of the following symptoms: cough, fever, wheezing, tachypnoea, general malaise, or loss of appetite.
- *Simple rhinitis:* Nasal discharge as the only symptom.
- *New episode*: An episode of ARTI or simple rhinitis with a 6-day interval free of symptoms to the previous same type of episode.
- *Time at risk:* Days with no recorded symptoms excluding the 6 consecutive days without symptoms following an episode.
- *Incidence:* Number of episodes divided by person time at risk.

4.4.3. Definition used in Paper IV:

- *Illness episode:* Time from first day with symptoms to last day with symptoms without symptom-free days in the interval. All symptoms occurring during the episode were considered associated with the hMPV or RSV infection.

4.5. Specimen collection

4.5.1. NPA (Paper II)

NPA specimens were taken using a soft plastic tube sucking secretions from the nasopharynx into a sterile 10 ml tube. Afterwards, two ml of phosphate-buffered saline (PBS) buffer were suctioned through the plastic tube, and the secretions were collected in the sterile tube. At home visits, the same device connected to a foot pump was used (Figure 3).

Figure 3. NPA collection devise connected to a foot pump.

4.5.1.1. Protein counts in NPAs (Paper II)

As only a small amount of secretion was obtained from asymptomatic children during the home visits, the total protein count in the NPA's was determined as a measure of sufficient sample material was obtained in each case. The protein concentrations in NPAs were determined by using the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL). Briefly, the assay is based on Bicinchoninic acid for detection and quantification of four amino acids, cysteine, cystine, tryptophan, and tyrosine within a sample. The obtained results were quantified relative to a standard curve based on bovine serum albumin at an optical density (OD) of 562 nm.

4.5.2. Nasal swabs (Paper IV)

Nasal swabs were obtained using a sterile cotton swab on an aluminium shaft, which was dipped into sterile sodium chloride before applied to the nose. Swabs were collected from a depth of 2 to 3 cm and were then placed into a vial containing 1 ml of viral transport medium (bovine serum albumin and antibiotics in PBS) (Figure 4 and 5). The aluminium shaft was chosen, as it could be bended in the preferable angle prior to collection. At some occasions the parents were instructed in sampling technique and sent the samples by mail to the laboratory.

Figure 4. Cotton swab and viral transport medium.

Figure 5. Collection of a nasal swab.

4.5.2.1. Validation of nasal swabs

The sensitivity of the nasal swabs compared with NPA was evaluated in 20 children aged 2 weeks to 6 months and hospitalised with RSV infection at the Department of Paediatrics, Hvidovre Hospital (Table 1).

Table 1. Results of real-time RT-PCR analyses of RSV RNA detected in nasopharyngeal aspirates, nasal swabs collected from a depth of 2-3 cm (nasal swabs), nasal swabs collected superficially from the nostrils (superficial swab), and nasal swabs stored at room temperature for 24 hours (swabs RT). All samples were collected from hospitalised children.

Child No.	NPA		Nasal swabs		Superficial swab		Swabs RT	
	result	Ct	result	Ct	result	Ct	result	Ct
1	pos*	na	pos	47.4	pos	>51	nd	na
2	pos	28.6	pos	34.0	pos	34.9	nd	na
3	pos	31.0	pos	43.1	pos	44.9	nd	na
4	pos	na	pos	32.5	pos	47.2	nd	na
5	nd	na	pos	31.0	pos	36.7	nd	na
6	pos	19.0	pos	26.9	pos	26.0	nd	na
7	pos	24.1	pos	32.5	pos	32.5	nd	na
8	pos	20.7	pos	28.6	pos	29.1	nd	na
9	pos	21.5	pos	31.1	pos	32.7	nd	na
10	nd	na	pos	28.8	pos	31.8	nd	na
11	pos	22.4	pos	26.2	pos	27.8	nd	na
12	pos	27.2	pos	27.1	pos	na	nd	na
13	pos	30.6	pos	31.9	nd	na	pos	32.0
14	pos	26.5	pos	34.4	nd	na	pos	37.6
15	pos	20.4	pos	22.2	nd	na	pos	22.5
16	pos	25.1	pos	36.1	nd	na	neg	na
17	pos	23.1	pos	23.4	nd	na	pos	23.8
18	pos	23.1	pos	28.9	nd	na	pos	32.2
19	pos	18.5	pos	20.3	nd	na	pos	22.8
20	pos*	na	pos	22.3	nd	na	pos	22.6

* These NPAs were tested by enzyme immunoassay.

NPA = nasopharyngeal aspirate, RT=room temperature, Ct=threshold cycle, nd = not done, na=not available.

Nasal swabs were taken within one day of the NPA. A swab was obtained from each nostril; in 12 children the first swab was taken very superficially and the second as described above. In 8 children, both swabs were collected as described above. One swab from each of these 8 children remained at room temperature (RT) until the next day, whereas the remaining swabs were stored at 4˚C immediately. In this way, it was tested if the depth of collection and time to cooling had a major impact on the results of the PCR analyses. Swabs were analysed for the presence of RSV RNA by real-time RT-PCR as described below. Except for one swab stored at RT for 24 hours, RSV RNA was present in all samples, although most nasal swabs contained lower amounts of viral RNA, illustrated by a higher threshold cycle (Ct) value in the swabs.

4.5.3. Saliva (Paper II)

Saliva was collected using the Oracol device (Malvern Medical Developments, UK), which is a cylindrical polystyrene sponge attached to a plastic stick designed to be used as a toothbrush (Figure 6).

Figure 6. Collection of saliva using the Oracol device.

4.5.4. Blood (Paper II, IV)

In the excretion study, 1-2 ml blood were collected from hospitalised children who had blood samples taken due to their illness.

In the birth cohort study, blood sampling from the children was performed as a heal prick together with routine PKU testing and as venous puncture at age 12 months. Blood samples from the mother were obtained at enrolment. Samples were taken to obtain exact information about serum RSV and hMPV antibody levels of the mother and child close to delivery and of the child by the age 12 months.

4.5.5. Sweat (Paper II)

Sweat was collected by pilocarpine iontophoresis (Figure 7). A disc of 0.5 % pilocarpine was applied to the medial aspect of the forearm after washing thoroughly with sterile water. After stimulation with iontophoresis for five minutes the arm was again cleaned with sterile water and sweat was collected through a closed spiral microbore tube (Figure 8).

4.5.6. Urine (Paper II)

Urine was collected using a plastic urine collector bag attached to the outer genitalia and then the urine was transferred to a sterile 10 ml glass.

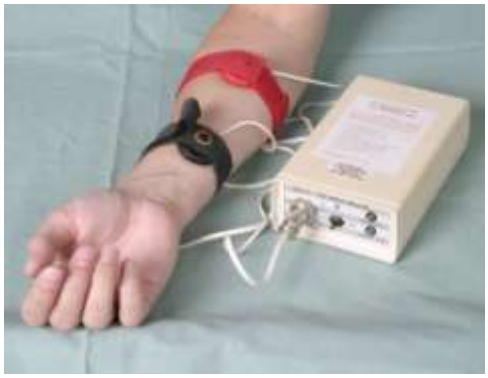

Figure 7. Pilocarpine iontophoresis. Stimulation of the forearm with iontophoresis. The red disc contains pilocarpine, and the black disc is a reference.

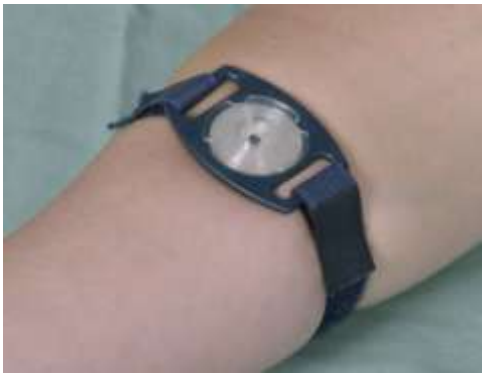

Figure 8. Collection of sweat through a closed spiral microbore tube, which is applied on the stimulated area for 30 minutes.

4.5.7. Faeces (Paper II)

Faeces were collected from the nappy by a sterile spoon and placed into a stool transportation tube containing 3-4 ml of Stool Transportation And Recovery (S.T.A.R.) buffer (Figure 9). The S.T.A.R buffer contains nucleases inactivators and permits storage and shipment of stool specimens for up to five days at 15-20°C without compromising the stability of RNA.

Figure 9. S.T.A.R buffer and stool collection device.

4.6. Real-time RT-PCR (Paper I, II, IV)

Nucleic acid purifications from NPAs, nasal swabs, saliva, urine and faeces specimens were carried out on a MagNA Pure LC Instrument. The amount of material used varied according to type of specimen. RNA from sweat and blood samples was extracted manually. Real-time RT-PCR

analyses of hMPV and RSV were performed on a LightCycler instrument (Paper I+II) or on a TaqMan PCR system (Paper IV).

In Paper I, commercially synthesized primers targeting the hMPV N gene were used for detection of hMPV RNA[87]. However, gradually it became clear that these primers only detected one subtype of hMPV. In the second paper, we used the same primers but added another primer set, which we were confident detected both subtypes of hMPV[90]. These were used in the fourth paper as well.

In Papers I and II, primers and fluorescence resonance energy transfer (FRET) probes were used for detection of RSV as described by Whiley et al[147]. In paper IV, primer/probe mixes for detection of RSV A and RSV B were ready-to-use mixtures obtained from Dr. Bert Niesters, Erasmus Medical Centre, Rotterdam, The Netherlands.

In all real-time RT-PCR analyses, standard precautions were taken throughout the PCR process to avoid possible cross and carry-over contamination. Positive and negative controls were included in purification and PCR procedures. For samples run on the TaqMan, Phocine Distemper Virus (PDV) was added to each sample prior to purification as an internal control. All hMPV and RSV-positive samples identified in Paper IV were tested for co-infection with rhinoviruses, adenovirus, influenza A and B, parainfluenzaviruses 1-3, coronaviruses OC43, 229E, NL63, and HKU1, and human bocavirus by PCR using the TaqMan system.

4.7. Enzyme-linked immunosorbent assay (ELISA) (Paper IV)

4.7.1. RSV ELISA

ELISA was used to determine anti-RSV IgG levels in plasma from mothers at the time of delivery and from their one-year-old children. Ninety-six-microwell, high-binding ELISA plates were coated with purified RSV antigen (8RSV79, HyTest, Finland) and incubated overnight at +4°C. The plates were washed with PBS containing Tween-20 and blocked with PBS containing 2% skim milk powder (M-PBS). Plates were washed, and serial dilutions of the positive control (3.86 mg/ml) were added together with plasma samples diluted in M-PBS and a negative control. Samples were measured in duplicate. After washing, plates were incubated with horseradish peroxidase (HRP)-conjugated goat-anti-human IgG, and antibodies were detected by adding 3,3´, 5,5´- tetramethyl benzidine (TMB) substrate. The reaction was stopped by using 1 M H_2SO_4 and the extinction was measured at 450 nm.

4.7.2. hMPV ELISA

hMPV antigen and positive and negative controls were obtained from Dr. Svein Arne Nordbø, Department of Medical Microbiology, Trondheim University Hospital, Trondheim, Norway. In Norway, hMPV antigen was prepared by sonicating infected LLC-MK2 cells in carbonate buffer. Sonicated cell pellet from the same cell line negative for hMPV was prepared the same way and used as negative control antigen. As positive and negative controls sera from two anti-hMPV IgG-positive persons and two anti-hMPV IgG-negative persons were used, respectively.

The ELISA plates were coated in every second well with hMPV antigen and negative control antigen, respectively, and incubated overnight at +4°C. The plates were washed with PBS containing Tween-20. Plasma samples and negative controls, diluted in M-PBS, were added in addition with serial dilutions of the positive control. After washing, plates were incubated with HRP-conjugated goat-anti-human IgG, and the presence of hMPV antibodies was detected by adding TMB substrate and then 1 M H_2SO_4 to stop the reaction. The extinction was measured at 450 nm with a reference filter at 630 nm. Net OD values were calculated as OD values for the antigen minus OD values for the control antigen. The cut-off value of each hMPV and RSV assay was defined by adding 2 standard deviations (SD) to the mean OD of all negative sera.

4.8. Phadiatop Infant analysis

Children in the birth cohort study were tested for the presence of allergen-specific IgE antibodies[5]. The analyses were performed by Phadia ApS, Denmark. Phadiatop Infant was analysed quantitatively in plasma, collected at one year of age, using Pharmacia CAP System™. The following allergens were included in the test; Egg white, cow's milk, peanut, *Dermatophagoides pteronysssinus*, cat, dog, birch, timothy, and mugwort. Values ≥ 0.35 Pharmacia Arbitrary Units per litre (PAU/l) were considered to indicate sensitisation and were coded as positive.

4.9. Sample size calculation (birth cohort study)

We assumed that 75% and 25% of the children became infected with RSV and hMPV, respectively, during the first year of life and that the cases were detected during the study period. With a chosen sample size of 200 subjects it would be possible to detect differences in symptoms related to RSV and hMPV infection with an odds ratio (OR) of 2.8 under the assumption of 5% significance level and 80% strength. Risk factors for RSV or hMPV infection during the first year of life could be detected with relative risks (RR) of 1.3 and 2.1, respectively, under the assumption that the actual

risk factors were time-independent and present in 50% of the population. Power calculations were made in EpiInfo 2000.

4.10. Statistical methods (Paper I, II, III, IV)

Differences in categorical data between the various groups of children were tested by using chi-square test or Fisher's exact test. For non-normally distributed continuous data, median, range, and in some cases inter quartile ranges (IQR), were calculated and compared by using the non-parametric Mann-Whitney U-test and the Kruskal-Wallis test. Shedding durations of RSV and hMPV RNA in NPAs were estimated by Kaplan-Meyer curves and differences were evaluated by a log rank test.

In Papers III and IV, logistic regression analyses were performed to assess the importance of various factors for dichotomic outcomes, and to calculate OR. To reduce problems with co-linearity, only 1-3 variables from each group of covariates were included. Effect modification was evaluated in logistic regression analyses with interaction terms.

In Paper III, logistic regression was used to model the daily episode status. For incidence only days at risk were modeled and for prevalence all observed days were included. Relevant information from interview schemes was carried forward two months. To account for the possible correlation between episodes from the same child, OR and confidence intervals (CI) were estimated by using generalised estimating equations (GEE) with an autoregressive correlation structure (AR(1)). P-values were calculated by using Wald's test.

All regression analyses in Paper III were adjusted for sex, age and calendar period. The calendar effect was modeled as a periodic function with a period of one year by inclusion of a sine and a cosine term in the models. The parameters for these two terms were transformed into parameters giving the time of maximum incidence/prevalence and the OR for December vs. July, respectively, and standard errors of these estimates were calculated.

Multiple logistic regression analyses were performed by including all the selected predictor variables in a model and successively removing the variable with the highest p-value, continuing until all predictors had a *p*-value below 0.1. A p-value<0.05 was considered significant. Depending

on the project, data were analysed by using the SAS software version 8e, SPSS version 12.0 or 13.0 for Windows, or R version 2.4.1 by using the geepack library.

4.11. Ethical considerations

The studies fulfilled the Helsinki Declaration II and were approved by The Ethics Committee of Frederiksberg, Copenhagen, Denmark (J. No. 01-028/03, 01-190/03, and 01-049/04), and by the Data Protection Board in Denmark (J. No. 2003-41-3092, 2003-41-3469, and 2004-41-4147).

5. Results

5.1. Paper I

In the first study, hMPV was identified in NPAs from Danish children hospitalised with ARTI, thereby proving that hMPV circulates in Denmark and may be a cause of respiratory illness. hMPV was detected in 11 (2.9%) and RSV in 190 (49.6%) of 383 samples. Two children were co-infected with both hMPV and RSV. These two children were not more severely ill than mono-infected children and were left out of statistical analyses. Age at admission was median 3.5 and 3.2 months for hMPV and RSV-positive children respectively. The peak incidence of hMPV infection was in February, approximately one month later than the RSV peak (Figure 10).

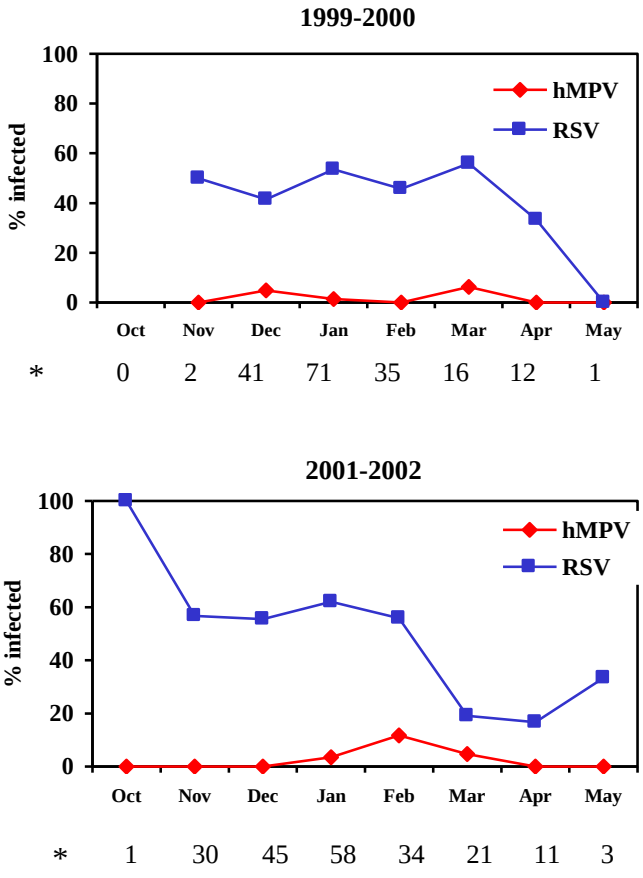

Figure 10. Seasonal distribution of hMPV and RSV-positive ARTI episodes during 2 winter seasons. *Total number of NPA examined per month.

Overall symptoms and clinical findings were similar among hMPV and RSV-positive episodes. However, no hMPV-infected children required treatment with n-CPAP or supplemental tube feeding in contrast to a quarter of the RSV-positive children (Table 2). The distribution of clinical diagnosis (LRTI or URTI) in hMPV and RSV-infected children was similar. However, when looking specifically at those of the children in the LRTI group with a diagnosis of asthmatic bronchitis, 6 (66.7%) of the hMPV-positive children received this diagnosis compared with 20 (10.6%) of the RSV-positive children ($p<0.001$) (Table 2).

Table 2. Symptoms, clinical findings, treatment, and main diagnoses in children hospitalised with respiratory tract infection, Copenhagen, 1999/2000 and 2001/2002

	hMPV (n=9)		RSV (n=188)		p-value
	No. positive/ total	(percent)	No. positive/ total	(percent)	hMPV vs. RSV
Symptoms and clinical signs					
Reported fever	6/8	(75.0)	127/184	(69.0)	1
Nasal discharge	6/6	(100)	145/151	(96.0)	1
Cough	8/8	(100)	175/175	(100)	-
Vomitus or diarrhoea	0/8	(0)	38/186	(20.4)	0.36
Tachypnoea	8/9	(88.9)	146/180	(81.1)	1
Chest indrawing	7/8	(75.0)	109/180	(60.6)	0.49
Rhonchi	7/9	(77.8)	88/187	(47.1)	0.09
Crepitation	1/9	(11.1)	77/187	(41.2)	0.09
Treatment					
Nasal CPAP	0/9	(0)	50/188	(26.6)	0.12
Nasal O_2	1/9	(11.1)	49/188	(26.1)	0.45
Assisted ventilation	0/9	(0)	2/188	(1.1)	1
Tube feeding	0/9	(0)	55/188	(29.3)	0.06
Main episode diagnosis					
Upper respiratory tract infection	1/9	(11.1)	38/188	(20.2)	0.7
Lower respiratory tract infection	8/9	(88.9)	145/188	(77.1)	0.7
Asthmatic bronchitis	6/9	(66.7)	20/188	(10.6)	<0.001

5.2. Paper II

In this study, 44 children aged 0.5-38 months were included (37 RSV-positive, 6 hMPV-positive, and 1 co-infected child). The hMPV and RSV-infected children did not differ significantly according to ethnicity, gender, duration of symptoms prior to admission, hospitalisation time, GA, chronic diseases, number of siblings, and smoking in household. Median age was 3.7 months for RSV-positive children and 7.8 months for hMPV-positive children ($p=0.1$).

Viral RNA was detected in saliva from 26/38 (68%) RSV-infected children and in 1/7 (14%) hMPV-infected children. Eighty-nine percent of the positive saliva samples were taken within the

first six days after diagnosis. RSV RNA was found in five stool samples from five different children. All positive samples were taken within two days of the diagnostic NPA, and four of the children had diarrhoea. hMPV RNA was not detected in stools. Three RSV-positive children (9%) and two hMPV-positive children (29%) shed viral RNA in sweat including the child with co-infection, who shed both RSV and hMPV in sweat. Children shedding RNA in sweat were either less than five weeks of age or had a chronic lung disease. We did not detect viral RNA in urine or blood samples.

Nine of 12 children shedding RSV RNA after 2 or 3 weeks had a negative RSV PCR test result in between. Melting point temperature analyses of NPAs from six of these children showed that they were infected with the same subtype of RSV (A or B) in all their positive samples. Assuming that all children shed RSV until the last positive sample, the median duration of RSV shedding was 11.5 days (Inter-quartile range (IQR) 6.5-18.5) while the median duration of hMPV shedding was 5.0 days (IQR 3.5-10.0) ($p=0.001$, Figure 11). There was a statistically significant association between protein count in the NPAs and symptoms at the time of sample collection ($p<0.0001$), between protein count and a positive test result ($p=0.01$), and between protein count and time of NPA collection ($p<0.01$). Anyhow, this could not explain all the negative test results in between two positive test results, as only three of nine children had the lowest protein count in their negative sample.

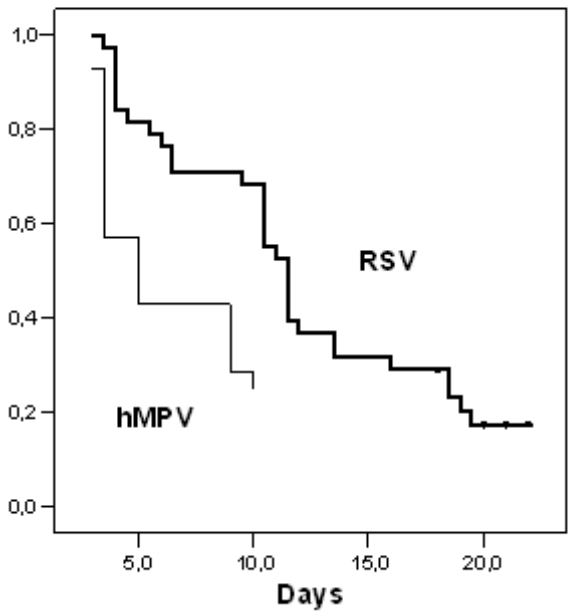

Figure 11. Kaplan Meyer analysis of shedding duration of RSV and hMPV RNA in nasopharyngeal aspirate specimens. RSV: median 11.5 days (IQR 6.5-18.5), hMPV: median 5.0 days (IQR 3.5-10.0), p=0.001

More than 75% of family members of both RSV and hMPV-infected children had a URTI when they were followed up. In families with more than one child, an older sibling was the first person to present symptoms in more than two thirds of the cases (75% for RSV and 60% for hMPV).

5.3. Paper III

Of 242 children whose parents accepted to participate in this study, 228 children were included in the analysis with an observation period of 80,013 days. Of these 228 children, 121 were boys and 105 had siblings. Twenty-three percent of the children lived with parents who smoked and 72% belonged to social class 1 or 2. Breastfeeding was initiated by 95% of the mothers. Only 22% of the infants attended daycare outside the home by the age of 10 months.

On average, children had one or more symptoms for 3.5 months during their first year of life, nasal discharge and cough being far most prevalent (Figure 12). Frequency of all symptoms increased steeply after six months of age. Each child had on average 6.3 episodes (median=5.1, IQR: 3.3-7.8) of ARTI and 5.6 episodes (median=4.3, IQR: 2.1-7.3) of simple rhinitis per 365 days at risk. Forty percent of simple rhinitis episodes proceeded into an ARTI episode. An average ARTI episode lasted for 4.7 days (median=3, IQR: 2-6).

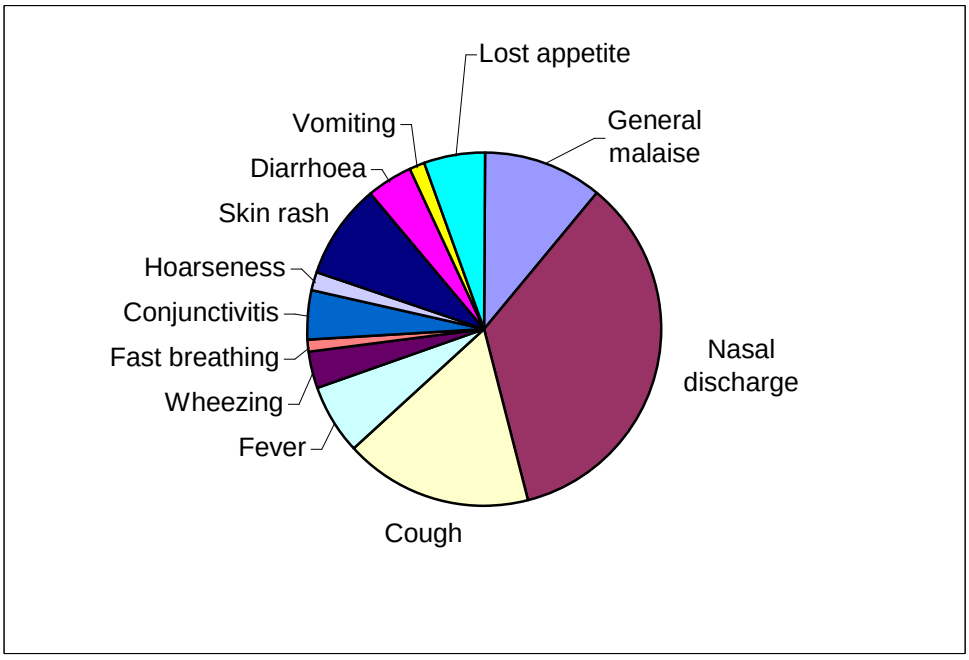

Figure 12. Percentage distribution of general symptoms.

The odds of developing ARTI and simple rhinitis increased significantly from six months of age, in the winter season, and when having siblings aged 1-3 years. Odds of an ARTI were increased for children attending large daycare centres, while large household size and living in small average rooms were associated with simple rhinitis (Table 3). The effects of age and season are illustrated in Figures 13 and 14.

Table 3. Multiple logistic regression analysis for incidence of acute respiratory tract infection (ARTI) and simple rhinitis. The effects of age and season are illustrated in Figure 13 and 14.

Variable	ARTI			Simple rhinitis		
	OR	95% CI	*p* value	OR	95% CI	*p* value
Siblings in day nursery (½-3 y)			0.01			<0.001
No	1.00	Reference		1.00	Reference	
Yes	1.41	(1.08-1.84)		1.99	(1.50-2.64)	
Each additional person in household	-	-	-	1.23	(1.10-1.38)	<0.001
Size of residence						0.03
Total m^2/no. of rooms > 30 m^2	-	-	-	1.00	Reference	
Total m^2/no. of rooms < 30 m^2				1.26	(1.02-1.56)	
Daycare attendance			0.02			
No	1.00	Reference		-	-	-
Family daycare home (2-4	1.46	(0.81-2.61)				
children)	1.49	(1.10-2.02)				
Daycare centre (10-15 children)						

Variables included in analyses are: age, gender, season, gestational age, mother's age, ethnicity, mother had a cold<2 weeks prior to delivery, siblings in daycare centre and day nursery, daycare attendance, socioeconomic status, and carpets. For ARTI included are in addition smoking inside, maternal smoking, breastfeeding, number of adults in the household, siblings, and atopy in siblings. For simple rhinitis included are in addition smoking during pregnancy, paternal education, exclusive breastfeeding, atopy in the family, moisture in home, size of residence, household size, and smoking in household. OR, odds ratio; CI, confidence interval

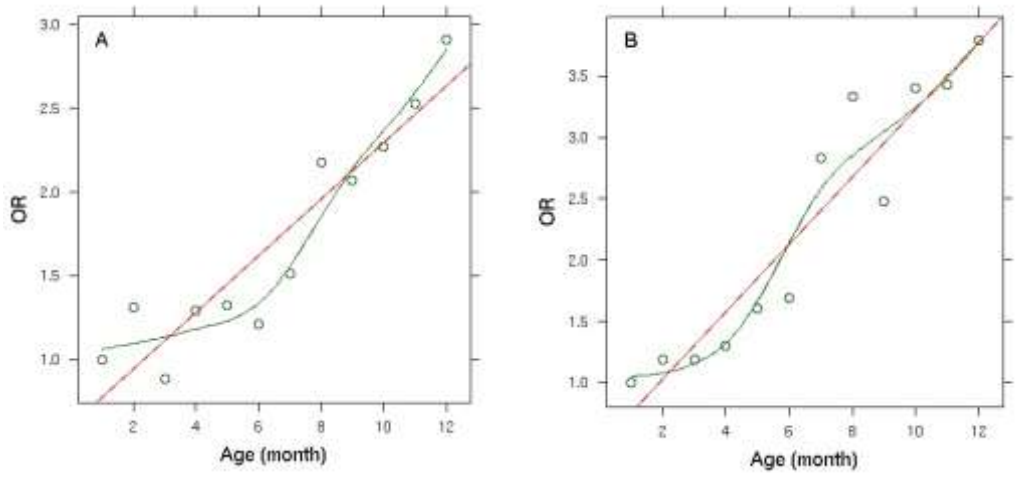

Figure 13. Multiple logistic regression analyses of age distribution of respiratory symptoms.

A: Incidence of simple rhinitis, B: Incidence of acute respiratory tract infection.

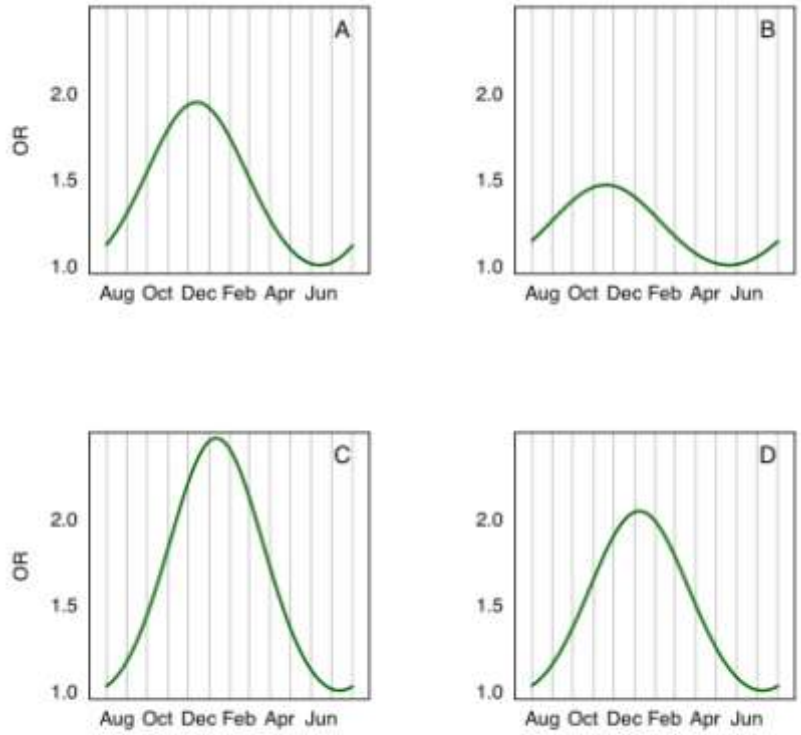

Figure 14. Multiple logistic regression analyses of seasonal distribution of respiratory symptoms. A: Incidence of simple rhinitis, B: Prevalence of simple rhinitis, C: Incidence of acute respiratory tract infection, D: Prevalence of acute respiratory tract infection.

5.4. Paper IV

Of 242 children whose parents accepted to participate in the study, 217 children were followed throughout one year and were included in the analysis.

Anti-hMPV IgG was found in 38 (17.5%) children and anti-RSV IgG in 172 (79%) children by age one year. Odds of being anti-hMPV IgG-positive were increased by a factor 2.36 (95% CI: 1.06-5.27) and 3.82 (95% CI: 1.75-8.34), respectively, in children born during the spring and in children with older siblings. Drying clothes inside was associated with a decreased risk of being anti-hMPV IgG-positive (OR=0.45, 95% CI: 0.21-0.97). Factors associated with being anti-RSV IgG-positive were: GA<38 weeks (OR=3.39; 95% CI: 1.42-8.05), increasing paternal age (OR=1.85 per five yrs;

95% CI: 1.28-2.68), and wall-to-wall carpeting (OR=3.15; 95% CI: 1.29-7.68), while birth into the spring decreased the odds of being anti-RSV IgG-positive (OR=0.27, 95% CI: 0.09-0.85). Sleeping outside during the day seemed to protect against RSV infection, however non-significantly (OR=0.44, 95% CI: 0.19-1.03). Risk factors for RSV hospitalisation (n=11) were: older siblings (OR=4.49; 95% CI: 1.08-18.73) and smoking in the household (OR=5.06; 95% CI: 1.36-18.76). Exclusive breastfeeding for the first 14 days of life was associated with a reduced risk of being hospitalised (OR=0.21, 95% CI: 0.06-0.79).

The time of primary infection was identified in 10 hMPV and 15 RSV infections. RSV-positive samples were identified from children down to 26 days of age, while primary hMPV infection was only seen in children older than six months of age. All possible symptoms from the health diaries occurred in both RSV and hMPV-infected children, although more frequently among the RSV-positive children (non-significant).

5.5 Phadiatop Infant

Phadiatop Infant was positive in 15 (7%) of the children at age 12 months. Nine children were IgE-sensitised to milk, seven to egg and one to peanut. Of these children, 40% had atopic dermatitis compared with 18% of all children (p=0.03). IgE-sensitisation was not associated with hMPV or RSV infection, respiratory symptoms, diarrhoea or atopic disposition.

6. Discussion

6.1. Study designs, data quality and validity

In the first study, we included all children who had been hospitalised with an ARTI during two winter seasons and for whom a routine NPA for RSV analysis had been stored. According to the result of the PCR analysis of the NPA, children were divided into three groups (hMPV-positive, RSV-positive, and hMPV/RSV-negative). Because children in each group were hospitalised with roughly the same symptoms, in the same hospitals, at the same period of time, the risk of selection bias was avoided.

In the excretion study, children were included when the result of the NPA test was available and followed three weeks from then. The results of shedding duration are therefore related to time of hospitalisation rather than to time of infection. Parents were interviewed weekly on symptoms in the child and the family minimizing the risk of recall bias. However, in a few cases, the parents reported the first date with symptoms in their child to be later than the date of hospitalisation, which may be interpreted as a recall bias. The main purpose of this study was to investigate the sites of viral excretion. However, when studying the prevalence of respiratory symptoms in family members it would have been appropriate to have a control group of families with RSV and hMPV-negative children to see if the same amount of respiratory illness occurred in such families.

The birth cohort study was designed to reduce the effects of chance and bias. The study was conducted over two years to reduce the impact of possible seasonal variation. Information on background variables was collected at the first home visit (after one month), and we used diary cards to be filled out on a daily basis, thereby minimizing recall bias[11, 142]. The active surveillance by monthly home visits ensured that any queries concerning the diaries were rectified, it helped maintain high compliance by parents and resulted in a low drop-out rate of 10.3% during one year. When looking specifically at the children actually participating in the study with at least one home visit, the drop-out rate was only 4.8%, which compares favourably with the 47% drop-out rate in an Australian study[25], where parents used a self-administered daily diary, and 11.9% in a Dutch prospective birth cohort study[141]. A recent Swiss birth cohort study assessing symptom frequency by weekly telephone interviews had a drop-out rate of only 4.9% during one year[78].

Parents who gave birth to their first child were generally more eager to participate in the birth cohort study because of the close contact to health professionals provided by monthly home visits. However, we were aware of that and attempted to include an equal number of only children and children with siblings to avoid selection bias. Likewise, families with very few resources were underrepresented, as participation in the study required cooperation in the form of keeping a diary and remembering the monthly appointments. In addition, some families might not like the idea of letting a stranger into their private home. Overall, the cohort represents infants from a city area with an overrepresentation of highly educated parents and a high rate of children being breastfed. The cohort is therefore not representative for the whole country, but results may be referred to other city areas in Denmark and to Western cities with a similar breastfeeding prevalence.

A sample size calculation showed that 200 children should be included in the study to be able to detect differences in symptomatology between hMPV and RSV infections with an OR of 2.8. However, this calculation was based on the assumption that all hMPV and RSV infections were identified during the study period. Due to resource constraints and as 89% of anti-hMPV IgG-positive children were also anti-RSV IgG-positive, we chose to analyse nasal swabs from the anti-hMPV IgG-positive children only to detect the time of primary hMPV or RSV infection. In this way, we identified 26.3% (10 out of 38) of the hMPV infections and 8.7% (15 out of 172) of the RSV infections, which were too few to detect differences – if any - in symptomatology. The sample size was sufficiently large to detect risk factors for hMPV and RSV infection with RR of 2.1 and 1.3 respectively, thereby reducing, however not eliminating, the possibility that findings may be due to chance. In order to control confounding we used multiple logistic regression analysis for estimating the magnitude of the association between the exposure and the outcome after adjusting for a number of potential confounding factors. Furthermore possible effect modifications were explored by generating interaction terms between some of the factors under investigation.

6.2. Specimen collection

In the excretion study, collection of nasal secretes was performed weekly and not daily, allowing only a rough estimate of shedding duration of viral RNA. Parents collected urine and faeces specimens from their child prior to our home visits, and occasionally, if samples were not ready at our visit, they were sent by mail to the laboratory. These samples were frozen approximately two days after collection, which might influence the stability of viral RNA in urine samples. However,

as S.T.A.R. buffer was added to the stool collection device, viral RNA in stool samples should not be affected by this. As blood was obtained only from children who had a blood sample taken due to their illness, no information on the presence of viral RNA in blood was available for half of the included children. For collection of oral fluid the Oracol device was chosen, taken into account acceptability, price, and quality[145].

Collection of sweat was done by using a device which is used routinously when testing children for cyctic fibrosis. To avoid the risk of contamination, we washed the arm thoroughly with sterile water prior to applying the pilocarpine disc and again prior to applying the spiral. We did not use ethanol for washing as RSV has a lipid membrane that is easily destroyed by alcohol, which could also affect the stability of RNA excreted in sweat[110]. It is only possible to collect a relatively small amount of sweat from young children, and freezing and thawing undiluted samples make the RNA very sensitive to degradation. This made it difficult to reproduce our findings by RT-PCR and due to lack of material we were not able to perform cell culture analyses and only succeeded in sequencing one sample.

Nasal swab offers a sensitive sampling method for the detection of respiratory viruses in children. RSV is an exception and it is detected more often in NPA than in nasal swabs when searched for by ELISA, IFA, or viral culture[56, 86, 130]. However, a recent study reports that when a sensitive amplification method like RT-PCR is used for RSV analysis, nasal swabs have a sensitivity, specificity, positive predictive value, and negative predictive value of 96%, 100%, 100%, and 91%, respectively[146]. The nasal swab specimens were chosen for surveillance of respiratory infections in the birth cohort study, as they were sensitive in our own test of RSV in hospitalised infants, as they are easy to collect, require no additional devices, and cause much less pain and distress than NPA[86]. As we collected nasal swabs at regular monthly intervals, nasal secretions from many ARTI episodes were not available. We used this method to avoid the risk of bias and underreporting by parents if they actively had to contact us for every respiratory episode, which has been the case in other studies[22].

6.3. Laboratory methods used in the studies

In Papers I, II, and IV, real-time RT-PCR was used for RNA detection. Except for sweat and blood, samples were extracted using the MagNA Pure LC Instrument. The PCR settings changed and different protocols were used in the studies, but for each assay published primer sequences and

adequate controls were used and standard precautions taken to avoid cross and carry-over contamination. Despite the fact that we used primers that only detected one subtype of hMPV in the first study, the set up of each assay is superior to any other detection method of RSV and hMPV.

ELISA was used in Paper IV for detection of anti-hMPV and anti-RSV IgG in plasma from mothers and children. Exact concentrations were determined for RSV IgG. We are not aware of the existence of a standardised hMPV antigen with a known concentration, and the standard curve in the hMPV ELISA was made from serial dilutions of a positive control without a known concentration. The OD values did not allow us to determine an exact concentration of anti-hMPV IgG, however, they could point in the direction of high or low IgG levels in a sample. As the primary goal was a qualitative measurement of IgG in the samples, this is not a major limitation of the assay. The inter- and intraassay variations of each assay were acceptable.

The Phadiatop Infant test was performed by Phadia ApS, Denmark. The diagnostic efficacy of the test has been evaluated in studies of IgE sensitisation in young children with and without clinical symptoms of atopic diseases[5, 36]. In a prospective birth cohort study of unselected children, the sensitivity of the Phadiatop Infant test was 96% among children characterised as IgE-sensitised (positive skin prick test (SPT) and allergen-specific IgE) and 82% if children with either a positive SPT or allergen-specific IgE were included. The specificity was 98%[5].

6.4. Discussion of findings in each study

6.4.1. Paper I

The detection of hMPV in 2.9% of the children hospitalised with ARTI is in the lower end of what has been reported in other studies of similar design[2, 6, 107, 127]. As already mentioned, the use of primer sequences detecting only one subtype of hMPV explains some of this lower prevalence. Studies in which only samples tested negative for all known respiratory viruses were analysed for hMPV naturally report higher frequencies of hMPV. Differences in patient types and sample methodology may also explain differences in hMPV frequencies.

We detected hMPV-positive samples from end of December to mid March, with a peak in February. As the study was designed to include NPAs collected during the winter season only, a seasonal distribution covering a whole year was not determined. Other studies have found hMPV infection to

occur mainly during the winter season, although positive samples are also found during the summer in contrast to RSV[113, 149].

Surprisingly, two thirds of the hMPV-infected children were discharged from hospital with a diagnosis of asthmatic bronchitis. This was significantly more than for RSV-infected children. RSV is in addition to rhinoviruses known to trigger asthma exacerbations in children and adults[16]. The relative role of hMPV in the induction of asthmatic phenotypes remains to be determined, but findings from other studies indicate that hMPV plays an important role in the pathogenesis of wheezing[63, 92, 107].

6.4.2. Paper II

We detected RSV RNA in faeces specimens from five children of whom four had diarrhoea. The study is the first to detect RSV RNA in faeces. Recently, other respiratory viruses, such as adenovirus[84], SARS-CoV[26], and human bocavirus[144], have been detected in stool. An explanation for the finding of RSV RNA in stools might be that the children have swallowed respiratory secretions containing RSV. The possibility that RSV replicates in the gastrointestinal tract seems less likely. We did not perform viral culture analyses of the stools to confirm infectivity of the viral RNA.

RSV and hMPV RNA were detected in sweat from four children who were under five weeks of age or had a chronic lung disease, indicating that an immature or defective immune response facilitates the spread of the virus from the upper respiratory tract. A short viraemic phase would allow the virus to spread to the sweat glands to be excreted or to replicate. Other groups have detected RSV and hMPV RNA in blood[91, 115], and recently rhinovirus viraemia has been described to occur in normal children with a respiratory infection[150]. No other groups have reported the detection or search for respiratory viruses in sweat, but SARS-CoV has been detected in sweat glands of the skin[23]. The Ct values of the RT-PCR analyses of sweat were high, indicating that only few copies of RNA were present in the samples. This makes high demands on the sensitivity of the detection method, and low amounts of viral RNA are not likely to be detected by less sensitive methods like IFA or ELISA. With the implementation of highly sensitive real-time RT-PCR systems in many research laboratories around the world it will be interesting to see what future research will add to our current understanding of viral excretion and transmission.

The median shedding durations of RSV and hMPV in nasal secretions were 11.5 and 5 days, respectively. A previous study found that the shedding duration of RSV in hospitalised children ranged from 1 to 21 days with a mean of 6.7 days measured by cell culture[45]. Those of the children with an LRTI shed virus for mean 8.4 days and those with a URTI for mean 1.5 days. No similar studies have been published for hMPV. Our results are based on findings by RT-PCR, which may be more sensitive than cell culture, explaining the longer shedding period in our study.

6.4.3. Paper III

Children in this study had one or more symptoms for 3.5 months during their first year of life. Not many studies of similar design have been conducted. Previous Danish studies on this topic are either cross-sectional, retrospective, focus mainly on older children, or follow children for a short period of time[53, 101, 102, 135]. Our findings are equal to the findings of a British longitudinal birth cohort study of infants' general symptoms[59], although we report higher prevalences of nasal symptoms, fever, and cough. The reported prevalence of respiratory symptoms in our study is higher than for most studies [25, 59, 78], except studies from Greenland reporting respiratory symptoms on 41.6% of the days of observation for children < 2 years of age[69].

We found that each child had on average 6.3 ARTI episodes per 365 days at risk. Including episodes with simple rhinitis, each child had in total 9.7 respiratory episodes per 365 days at risk. Most other studies report mean numbers of ARTI between 3.8 and 6.9 during the first year of life[4, 7, 25, 60, 74, 81, 85, 96], although higher incidences are reported from Greenland[69] and Mozambique[114]. However, any direct comparisons between these studies and ours are difficult because of differences in design, climate, demographics of the populations studied, definitions and classifications of respiratory illness, the period required between episodes, and the methods of surveillance employed.

The most substantial risk factors for respiratory symptoms in our study were increasing age, winter season, and exposure to infections reflected by having siblings in a day nursery, number of household members, living in small average rooms, and daycare attendance. The amount of disease was relatively low in children under six months of age, after which a steep rise in respiratory symptoms occurred. With increasing age several factors arise which influence on the child's

susceptibility to infections, such as degradation of maternal antibodies, cessation of breastfeeding, coming into more contact with others, and start at daycare centres at a time when the adaptive immune system is still immature. Household size and size of residence were in our study only associated with risk of simple rhinitis, indicating that crowding in the home, however important, is mainly related to mild symptoms, while daycare attendance has a much higher impact on the severity of respiratory illness. An important risk factor for respiratory symptoms was having siblings aged 1-3 years. This is in accordance with other studies reporting that the number and age of siblings predict the incidence of lower respiratory illness and wheezing in infancy[25, 78, 82, 122].

6.4.4. Paper IV

In this study, we found that risk factors for hMPV and RSV infection were different, as were risk factors for mild and severe RSV infection. The study is the first to identify risk factors for primary hMPV infection; presence of older siblings and being born in the spring. No other studies are directly comparable with ours, but studies including children with underlying pathologies have found prematurity, male gender, congenital heart disease and gastrointestinal reflux or aspiration to be risk factors for hMPV hospitalisation[113]. The finding that drying clothes inside protects against hMPV infection is difficult to explain and may be due to chance.

We identified several factors, which were associated with RSV infection and hospitalisation. Some risk factors were well known from previous studies (low GA, siblings, smoking, and lack of breastfeeding[15, 77, 103, 112]), while others were new to us: Increasing paternal age and wall-to-wall carpeting. With increasing age, antibody levels decline, making the individual more susceptible to new infections. An explanation could be that older fathers due to low levels of RSV antibodies become infected and transmit the virus to the child. However, we did not collect plasma samples from the fathers for antibody detection to confirm this theory.

The significance of carpeting is controversial with respect to allergic diseases; most studies report carpeting to be associated with higher levels of house dust mites, thus aggravating the severity of allergic asthma[109, 139]. To our knowledge, the association between carpeting and RSV infection has not been described previously. It is not known whether the survival of RSV is greater on carpets than on smooth floors, but young children spend much time lying and crawling on the floor, and transmission from surfaces contaminated with RSV-infected nasal secretions is possible[45]. Maybe parents are more inclined to place their child directly on a carpet rather than on a cold floor, and

maybe cleaning is easier achieved on the floor through rubbing. We found a non-significant tendency of a protective effect of sleeping outside during the day. However, the sample size may have been too small to reach statistical significance and further studies are warranted to confirm this finding.

6.4.5. IgE-sensitisation

There is good evidence from clinical and experimental studies of RSV that LRTI may contribute to early systemic sensitisation to common allergens[104, 119]. Fifteen (7%) of the children in our study had allergen-specific IgE detected in their blood at age 12 months, which is to be expected in a cohort of healthy 1-year-old children[61]. A positive test was associated with the presence of atopic dermatitis, but not with hMPV or RSV infection, respiratory symptoms or diarrhoea, findings that are in accordance with a recent Swedish study[5]. Low levels of specific IgE to food allergens are a common phenomenon during early childhood and may have no clinical importance. However, the presence of IgE antibodies against hen's egg during early infancy has been shown to predict respiratory allergy[100]. A follow-up of this cohort, when the children reach school age, will show whether these cases of low-grade IgE sensitisation at young ages are only temporary and without clinical importance, or whether they have an impact on the development of allergic phenotypes at older ages.

7. Conclusion and perspectives

Conclusion

The studies in the present thesis provide detailed data on the occurrence of ARTI in Danish infants and verify that hMPV is present in young Danish children with ARTI, although less frequent than RSV and with a tendency to a milder clinical course. hMPV and RSV have tropism for respiratory epithelium cells, however, we detected viral RNA in faeces and sweat, indicating a systemic spread of virus. Respiratory illness was associated with increasing age, winter season, household size, daycare attendance, and having young siblings. Risk factors for early hMPV and RSV infection and RSV hospitalisation were different; passive smoking and older siblings being determinants for a severe outcome (i.e. hospitalisation).

Perspectives

Risk factors identified to be associated with ARTI in this study confirm the heterogeneity in the factors as well as the limited potential for intervention. However, a reduction of illness might be achieved by reducing transmission through improved hand hygiene practices in the homes and in daycare institutions. Denmark still has a high prevalence of smokers compared with other European countries, and public health policies must emphasize the harmful effects of passive smoking. Further studies are warranted to define risk factors for severe hMPV infection. In addition, clinical and virological studies are needed to elucidate the distribution of hMPV and RSV in different organ systems and bodily fluids during infection and to determine whether viral RNA excreted in non-respiratory secretions is infectious.

Complete elimination of the spread of RSV and hMPV is not possible, and prevention of disease by vaccines may be the ultimate goal. Efforts are ongoing to generate an effective and safe vaccine against RSV, and several candidate hMPV vaccines are under development. However, many fundamental questions concerning the pathogenesis of hMPV disease and the host's specific immune response need to be answered before an effective vaccine strategy for hMPV can be defined.

8. Summary

Acute respiratory tract infection (ARTI) in infants and young children is a significant public health problem worldwide. Respiratory syncytial virus (RSV) is the leading cause of lower respiratory tract infection in young children, but in many cases the etiological agent remains unknown. In 2001, a hitherto unknown respiratory virus - human metapneumovirus (hMPV) - was identified in the Netherlands. The discovery of hMPV forms the basis of the present thesis. The overall aim was to study the clinical epidemiology of hMPV compared with RSV in hospitalised and non-hospitalised children and to determine the frequency of acute respiratory symptoms in healthy infants. The thesis includes four papers based on three studies:

In the first study, we examined the frequencies and clinical features of hMPV and RSV infections in children hospitalised with ARTI. Nearly 400 nasopharyngeal aspirates from hospitalised children were analysed for hMPV and RSV. The presence of hMPV among Danish children was confirmed with the detection of 11 hMPV-positive samples and 190 RSV-positive samples. The symptoms associated with hMPV and RSV infection were very much alike, however, more RSV-positive children needed respiratory support. Asthmatic bronchitis was diagnosed more often in hMPV-infected children.

In the second study, the excretion patterns of hMPV and RSV were investigated. During 3 weeks, 7 hMPV-positive and 38 RSV-positive children had 4 nasopharyngeal aspirates, saliva, faeces, and urine samples taken in addition to 1 blood and 1 sweat sample. hMPV RNA was detected in saliva and sweat. RSV RNA was found in saliva, faeces, and sweat. Children shedding viral RNA in sweat were either younger than five weeks of age or had a chronic lung disease. Shedding durations for hMPV and RSV were 5 and 11.5 days, respectively.

In the third study, a birth cohort of 228 healthy infants was followed for 12 months with monthly home visits. Symptom diaries and nasal swabs were collected at every visit. Children had one or more symptoms for 3.5 months during the first year of life, including runny nose for 2 months. Each child had on average 6.3 episodes of ARTI (nasal discharge and ≥ 1 of the following

symptoms: cough, fever, wheezing, tachypnoea, malaise, or lost appetite) and 5.6 episodes of simple rhinitis per 365 days at risk. Risk factors for respiratory symptoms were: increasing age, winter season, household size, daycare attendance, and having siblings in a day nursery.

By age one year, 17.5% and 79% of the children had antibodies to hMPV and RSV, respectively. Risk factors for acquired hMPV infection were: being born during the spring and having older siblings. Risk factors for acquired RSV infection were: gestational age < 38 weeks, increasing paternal age, and wall-to-wall carpeting, while being born during the spring protected against infection. Risk factors for RSV hospitalisation were: older siblings and smoking in the household, while exclusive breastfeeding for the first 14 days of life protected against hospitalisation.

In summary, these studies provide detailed data on the occurrence of ARTI in young Danish children and verify that hMPV and RSV contribute to these infections. Symptoms associated with hMPV and RSV infection are similar among both hospitalised and non-hospitalised children. However, more RSV-infected children require respiratory support. Viral RNA is shed in faeces and sweat indicating a systemic spread of virus. Risk factors identified to be associated with ARTI in this study confirm the heterogeneity in the factors as well as the limited potential for intervention. The burden of ARTI caused by viral pathogens is impressive and leaves no doubt that effective and affordable vaccines are urgently needed.

9. Resumé på dansk

Luftvejsinfektioner hos små børn udgør et betydeligt globalt sundhedsproblem. Respiratorisk syncytial virus (RSV) er den hyppigste årsag til nedre luftvejsinfektion hos mindre børn, men i mange tilfælde forbliver det ætiologiske agens ukendt. I 2001 blev et nyt luftvejsvirus, human metapneumovirus (hMPV), identificeret i Holland. Udgangspunktet for dette ph.d.-studie var opdagelsen af hMPV. Hovedformålet med studiet var at belyse epidemiologien af hMPV sammenlignet med RSV hos hospitaliserede og ikke-hospitaliserede børn og at undersøge forekomsten af luftvejsinfektioner hos raske spædbørn. Afhandlingen indeholder 4 artikler baseret på 3 studier:

I første studie blev forekomsten og de kliniske symptomer ved en hMPV og RSV infektion undersøgt hos børn indlagt med akut luftvejsinfektion. Næsten 400 nasopharyngeal aspirater fra indlagte børn blev undersøgt for hMPV og RSV. Forekomsten af hMPV hos danske børn blev bekræftet med fundet af 11 hMPV-positive prøver og 190 RSV-positive prøver. Symptomerne ved de 2 typer infektion var meget ens, men flere RSV-positive børn krævede respiratorisk støtte. hMPV infektion var i højere grad end RSV infektion associeret med astmatisk bronkitis.

I andet studie blev udskillelsesmønstrene for hMPV og RSV undersøgt. Fra 7 hMPV-positive og 38 RSV-positive børn blev der over 3 uger opsamlet 4 nasopharyngeal aspirater, spyt, fæces- og urinprøver samt en blod- og en svedprøve. hMPV RNA fandtes i spyt og sved. RSV RNA fandtes i spyt, fæces og sved. Børn som udskilte viralt RNA i sved var enten yngre end 5 uger eller havde en kronisk lungesygdom. Udskillelsesvarigheden for hMPV og RSV i nasal sekret var henholdsvis 5 og 11,5 dage.

I tredje studie fulgte vi en fødselskohorte på 228 raske børn med månedlige hjemmebesøg indtil 1-årsalderen. Symptomdagbøger og næsepodninger blev indsamlet ved hvert besøg. Børnene havde et eller flere symptomer i 3½ måned det første leveår, heraf forkølelse i 2 måneder. Hvert barn havde i gennemsnit 6,3 episoder med luftvejsinfektion (løbenæse + hoste, feber, hvæsen, hurtig vejrtrækning, pjevset eller nedsat appetit) og 5,6 episoder med løbenæse. Risikofaktorer for

luftvejsinfektion og løbenæse var: stigende alder, vintersæson, stor husstand, at gå i daginstitution og at have søskende i vuggestue.

Henholdsvis 17,5% og 79% af børnene havde antistoffer mod hMPV og RSV ved 1-årsalderen som tegn på erhvervet infektion. Risikofaktorer for hMPV infektion var: født i foråret og at have ældre søskende. Risikofaktorer for RSV infektion var: født før fulde 38 uger, stigende fars alder og væg-til-væg tæpper i boligen, mens at være født i foråret beskyttede mod smitte. Risikofaktorer for at blive indlagt med RSV infektion var: ældre søskende og passiv rygning, mens fuld amning de første 14 dage beskyttede mod indlæggelse.

Sammenfattende giver studierne en detaljeret beskrivelse af forekomsten af luftvejsinfektion hos danske småbørn, og viser at både hMPV og RSV forårsager en del af disse infektioner. Symptomerne ved en hMPV og en RSV infektion er meget ens hos både indlagte og ikke-indlagte børn, men børn med RSV infektion kræver oftere respiratorisk støtte. Viralt RNA udskilles i sved og fæces tydende på en systemisk spredning af virus. Studierne bekræfter heterogeniteten af risikofaktorer for luftvejsinfektion og de begrænsede muligheder for intervention. Sygdomsbyrden ved virale luftvejsinfektioner er betydelig og understreger behovet for effektive og tilgængelige vacciner.

10. References

1. Abels S, Nadal D, Stroehle A, Bossart W. Reliable detection of respiratory syncytial virus infection in children for adequate hospital infection control management. J Clin Microbiol 2001;39(9):3135-3139.

2. Al-Sonboli N, Hart CA, Al-Aeryani A, Banajeh SM, Al-Aghbari N, Dove W, Cuevas LE. Respiratory syncytial virus and human metapneumovirus in children with acute respiratory infections in Yemen. Pediatr Infect Dis J 2005;24(8):734-736.

3. Alvarez R, Harrod KS, Shieh WJ, Zaki S, Tripp RA. Human metapneumovirus persists in BALB/c mice despite the presence of neutralizing antibodies. J Virol 2004;78(24):14003-14011.

4. Badger GF, Dingle JH, Feller AE, Hodges RG, Jordan WS, Jr., Rammelkamp CH, Jr. A study of illness in a group of Cleveland families. II. Incidence of the common respiratory diseases. Am J Hyg 1953;58(1):31-40.

5. Ballardini N, Nilsson C, Nilsson M, Lilja G. ImmunoCAP Phadiatop Infant - a new blood test for detecting IgE sensitisation in children at 2 years of age. Allergy 2006;61(3):337-343.

6. Boivin G, De Serras G, Côté S, Gilca R, Abed Y, Rochette L, Bergeron MG, Déry P. Human metapneumovirus infections in hospitalized children. Emerg Infect Dis 2003;9(6):634-640.

7. Borrero I, Fajardo L, Bedoya A, Zea A, Carmona F, de Borrero MF. Acute respiratory tract infections among a birth cohort of children from Cali, Colombia, who were studied through 17 months of age. Rev Infect Dis 1990;12 Suppl 8:S950-S956.

8. Bosis S, Esposito S, Niesters HG, Crovari P, Osterhaus AD, Principi N. Impact of human metapneumovirus in childhood: comparison with respiratory syncytial virus and influenza viruses. J Med Virol 2005;75(1):101-104.

9. Bouscambert-Duchamp M, Lina B, Trompette A, Moret H, Motte J, Andreoletti L. Detection of human metapneumovirus RNA sequences in nasopharyngeal aspirates of young French children with acute bronchiolitis by real-time reverse transcriptase PCR and phylogenetic analysis. J Clin Microbiol 2005;43(3):1411-1414.

10. Bulut Y, Guven M, Otlu B, Yenisehirli G, Aladag I, Eyibilen A, Dogru S. Acute otitis media and respiratory viruses. Eur J Pediatr 2007;166(3):223-228.

11. Butz A. Use of health diaries in pediatric research. J Pediatr Health Care 2004;18(5):262-263.

12. Carabin H, Gyorkos TW, Soto JC, Joseph L, Payment P, Collet JP. Effectiveness of a training program in reducing infections in toddlers attending day care centers. Epidemiology 1999;10(3):219-227.

13. Casiano-Colon AE, Hulbert BB, Mayer TK, Walsh EE, Falsey AR. Lack of sensitivity of rapid antigen tests for the diagnosis of respiratory syncytial virus infection in adults. J Clin Virol 2003;28(2):169-174.

14. Chanock R, Roizman B, Myers R. Recovery from infants with respiratory illness of a virus related to chimpanzee coryza agent (CCA). I. Isolation, properties and characterization. Am J Hyg 1957;66(3):281-290.

15. Cilla G, Sarasua A, Montes M, Arostegui N, Vicente D, Perez-Yarza E, Perez-Trallero E. Risk factors for hospitalization due to respiratory syncytial virus infection among infants in the Basque Country, Spain. Epidemiol Infect 2006;134(3):506-513.

16. Cohen L, Castro M. The role of viral respiratory infections in the pathogenesis and exacerbation of asthma. Semin Respir Infect 2003;18(1):3-8.

17. Collins PL, Chanock RM, Murphy BR. Respiratory syncytial virus. In: Knipe DM, Howley PM, eds. Fields virology. Philadelphia: Lippincott Williams & Wilkins, 2001:1443-1485.

18. Crowe JE, Jr., Williams JV. Immunology of viral respiratory tract infection in infancy. Paediatr Respir Rev 2003;4(2):112-119.

19. Cushing AH, Samet JM, Lambert WE, Skipper BJ, Hunt WC, Young SA, McLaren LC. Breastfeeding reduces risk of respiratory illness in infants. Am J Epidemiol 1998;147(9):863-870.

20. Danmarks statistik. Befolkningens uddannelse og beskæftigelse (Danish). www.dst.dk / September 2007.

21. Darville T, Yamauchi T. Respiratory syncytial virus. Pediatr Rev 1998;19(2):55-61.

22. de Waal L, Koopman LP, van Benten IJ, Brandenburg AH, Mulder PG, de Swart RL, Fokkens WJ, Neijens HJ, Osterhaus AD. Moderate local and systemic respiratory syncytial virus-specific T-cell responses upon mild or subclinical RSV infection. J Med Virol 2003;70(2):309-318.

23. Ding Y, He L, Zhang Q, Huang Z, Che X, Hou J, Wang H, Shen H, Qiu L, Li Z, Geng J, Cai J, Han H, Li X, Kang W, Weng D, Liang P, Jiang S. Organ distribution of severe acute respiratory syndrome (SARS) associated coronavirus (SARS-CoV) in SARS patients: implications for pathogenesis and virus transmission pathways. J Pathol 2004;203(2):622-630.

24. Døllner H, Risnes K, Radtke A, Nordbø SA. Outbreak of human metapneumovirus infection in Norwegian children. Pediatr Infect Dis J 2004;23(5):436-440.

25. Douglas RM, Woodward A, Miles H, Buetow S, Morris D. A prospective study of proneness to acute respiratory illness in the first two years of life. Int J Epidemiol 1994;23(4):818-826.

26. Drosten C, Gunther S, Preiser W, van der Werf S, Brodt HR, Becker S, Rabenau H, Panning M, Kolesnikova L, Fouchier RA, Berger A, Burguiere AM, Cinatl J, Eickmann M, Escriou N, Grywna K, Kramme S, Manuguerra JC, Muller S, Rickerts V, Sturmer M, Vieth S, Klenk HD, Osterhaus AD, Schmitz H, Doerr HW. Identification of a Novel Coronavirus in Patients with Severe Acute Respiratory Syndrome. N Engl J Med 2003;348(20):1967-1976.

27. Ebihara T, Endo R, Kikuta H, Ishiguro N, Ishiko H, Hara M, Takahashi Y, Kobayashi K. Human metapneumovirus infection in Japanese children. J Clin Microbiol 2004;42(1):126-132.

28. Ebihara T, Endo R, Kikuta H, Ishiguro N, Yoshioka M, Ma X, Kobayashi K. Seroprevalence of human metapneumovirus in Japan. J Med Virol 2003;70(2):281-283.

29. 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(3):1138-1141.

30. Eisenhut M. Extrapulmonary manifestations of severe respiratory syncytial virus infection--a systematic review. Crit Care 2006;10(4):R107.

31. 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(6 Pt 1):1407-1410.

32. Esper F, Martinello RA, Boucher D, Weibel C, Ferguson D, Landry ML, Kahn JS. A 1-year experience with human metapneumovirus in children aged <5 years. J Infect Dis 2004;189(8):1388-1396.

33. Falsey AR, Criddle MC, Walsh EE. Detection of respiratory syncytial virus and human metapneumovirus by reverse transcription polymerase chain reaction in adults with and without respiratory illness. J Clin Virol 2006;35(1):46-50.

34. Falsey AR, Formica MA, Walsh EE. Diagnosis of respiratory syncytial virus infection: comparison of reverse transcription-PCR to viral culture and serology in adults with respiratory illness. J Clin Microbiol 2002;40(3):817-820.

35. Falsey AR, Hennessey PA, Formica MA, Cox C, Walsh EE. Respiratory Syncytial Virus Infection in Elderly and High-Risk Adults. The New England Journal of Medicine 2005;352(17):1749-1759.

36. Fiocchi A, Besana R, Ryden AC, Terracciano L, Andreotti M, Arrigoni S, Martelli A. Differential diagnosis of IgE-mediated allergy in young children with wheezing or eczema symptoms using a single blood test. Ann Allergy Asthma Immunol 2004;93(4):328-333.

37. Fixler DE. Respiratory syncytial virus infection in children with congenital heart disease: a review. Pediatr Cardiol 1996;17(3):163-168.

38. Forster J, Tacke U, Krebs H, Streckert HJ, Werchau H, Bergmann RL, Schulz J, Lau S, Wahn U. Respiratory syncytial virus infection: its role in aeroallergen sensitization during the first two years of life. Pediatr Allergy Immunol 1996;7(2):55-60.

39. Freymouth F, Vabret A, Legrand L, Eterradossi 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(1):92-94.

40. García-García ML, Calvo C, Martín F, Pérez-Breña P, Acosta B, Casas I. Human metapneumovirus infections in hospitalised infants in Spain. Arch Dis Child 2006;91(4):290-295.

41. Glaser CA, Honarmand S, Anderson LJ, Schnurr DP, Forghani B, Cossen CK, Schuster FL, Christie LJ, Tureen JH. Beyond viruses: clinical profiles and etiologies associated with encephalitis. Clin Infect Dis 2006;43(12):1565-1577.

42. Glezen WP, Taber LH, Frank AL, Kasel JA. Risk of primary infection and reinfection with respiratory syncytial virus. Am J Dis Child 1986;140(6):543-546.

43. Graham NM. The epidemiology of acute respiratory infections in children and adults: a global perspective. Epidemiol Rev 1990;12:149-178.

44. Groth C, von Linstow ML, Høgh B. Behandling af bronchiolitis hos børn [Treatment of bronchiolitis in children] (Danish). Ugeskr Laeger 2007;In press.

45. Hall CB. The shedding and spreading of respiratory syncytial virus. Pediatr Res 1977;11(3 Pt 2):236-239.

46. Hall CB, Douglas RG, Jr. Modes of transmission of respiratory syncytial virus. J Pediatr 1981;99(1):100-103.

47. Hall CB, Douglas RG, Jr., Geiman JM. Respiratory syncytial virus infections in infants: quantitation and duration of shedding. J Pediatr 1976;89(1):11-15.

48. Hall CB, Douglas RG, Jr., Geiman JM. Possible transmission by fomites of respiratory syncytial virus. J Infect Dis 1980;141(1):98-102.

49. 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(8):414-419.

50. Hall CB, Powell KR, MacDonald NE, Gala CL, Menegus ME, Suffin SC, Cohen HJ. Respiratory syncytial viral infection in children with compromised immune function. N Engl J Med 1986;315(2):77-81.

51. Hall CB, Walsh EE, Long CE, Schnabel KC. Immunity to and frequency of reinfection with respiratory syncytial virus. J Infect Dis 1991;163(4):693-698.

52. Hall CB, Walsh EE, Schnabel KC, Long CE, McConnochie KM, Hildreth SW, Anderson LJ. Occurrence of groups A and B of respiratory syncytial virus over 15 years: associated epidemiologic and clinical characteristics in hospitalized and ambulatory children. J Infect Dis 1990;162(6):1283-1290.

53. Hansen BW. Acute illnesses in children. A description and analysis of the cumulative incidence proportion. Scand J Prim Health Care 1993;11(3):202-206.

54. Hansen EJ. Socialgrupper i Danmark (Danish). Socialforskningsinstituttet. Studie 48. 1984.

55. Hayes EB, Hurwitz ES, Schonberger LB, Anderson LJ. Respiratory syncytial virus outbreak on American Samoa. Evaluation of risk factors. Am J Dis Child 1989;143(3):316-321.

56. Heikkinen T, Marttila J, Salmi AA, Ruuskanen O. Nasal swab versus nasopharyngeal aspirate for isolation of respiratory viruses. J Clin Microbiol 2002;40(11):4337-4339.

57. Henderson FW, Collier AM, Clyde WA, Jr., Denny FW. Respiratory-syncytial-virus infections, reinfections and immunity. A prospective, longitudinal study in young children. N Engl J Med 1979;300(10):530-534.

58. 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;133(11):1135-1151.

59. Holme CO. Incidence and prevalence of non-specific symptoms and behavioural changes in infants under the age of two years. Br J Gen Pract 1995;45(391):65-69.

60. Hortal M, Benitez A, Contera M, Etorena P, Montano A, Meny M. A community-based study of acute respiratory tract infections in children in Uruguay. Rev Infect Dis 1990;12 Suppl 8:S966-S973.

61. Høst A, Halken S. The role of allergy in childhood asthma. Allergy 2000;55(7):600-608.

62. Isaacs D, Dickson H, O'Callaghan C, Sheaves R, Winter A, Moxon ER. Handwashing and cohorting in prevention of hospital acquired infections with respiratory syncytial virus. Arch Dis Child 1991;66(2):227-231.

63. Jartti T, van den Hoogen BG, Garofalo RP, Osterhaus AD, Ruuskanen O. Metapneumovirus and acute wheezing in children. Lancet 2002;360(9343):1393-1394.

64. Juntti H, Kokkonen J, Dunder T, Renko M, Niinimaki A, Uhari M. Association of an early respiratory syncytial virus infection and atopic allergy. Allergy 2003;58(9):878-884.

65. Kahn JS. Human metapneumovirus: a newly emerging respiratory pathogen. Curr Opin Infect Dis 2003;16(3):255-258.

66. Kaida A, Iritani N, Kubo H, Shiomi M, Kohdera U, Murakami T. Seasonal distribution and phylogenetic analysis of human metapneumovirus among children in Osaka City, Japan. J Clin Virol 2006;35(4):394-399.

67. Kamper-Jørgensen M, Wohlfahrt J, Simonsen J, Grønbæk M, Benn CS. Population-based study of the impact of childcare attendance on hospitalizations for acute respiratory infections. Pediatrics 2006;118(4):1439-1446.

68. Koch A, Mølbak K, Homøe P, Sørensen P, Hjuler T, Olesen ME, Pejl J, Pedersen FK, Olsen OR, Melbye M. Risk factors for acute respiratory tract infections in young Greenlandic children. Am J Epidemiol 2003;158(4):374-384.

69. Koch A, Sørensen P, Homøe P, Mølbak K, Pedersen FK, Mortensen T, Elberling H, Eriksen AM, Olsen OR, Melbye M. Population-based study of acute respiratory infections in children, Greenland. Emerg Infect Dis 2002;8(6):586-593.

70. Konig B, Konig W, Arnold R, Werchau H, Ihorst G, Forster J. Prospective study of human metapneumovirus infection in children less than 3 years of age. J Clin Microbiol 2004;42(10):4632-4635.

71. Korppi M, Piippo-Savolainen E, Korhonen K, Remes S. Respiratory morbidity 20 years after RSV infection in infancy. Pediatr Pulmonol 2004;38(2):155-160.

72. Kristensen K, Dahm T, Frederiksen PS, Ibsen J, Iyore E, Jensen AM, Kjær BB, Olofsson K, Pedersen P, Poulsen S. Epidemiology of respiratory syncytial virus infection requiring hospitalization in East Denmark. Pediatr Infect Dis J 1998;17(11):996-1000.

73. 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 lesions throughout the respiratory tract. Am J Pathol 2004;164(6):1893-1900.

74. Kusel MM, de Klerk NH, Holt PG, Kebadze T, Johnston SL, Sly PD. Role of respiratory viruses in acute upper and lower respiratory tract illness in the first year of life: a birth cohort study. Pediatr Infect Dis J 2006;25(8):680-686.

75. Kuypers J, Wright N, Corey L, Morrow R. Detection and quantification of human metapneumovirus in pediatric specimens by real-time RT-PCR. J Clin Virol 2005;33(4):299-305.

76. Kuypers J, Wright N, Morrow R. Evaluation of quantitative and type-specific real-time RT-PCR assays for detection of respiratory syncytial virus in respiratory specimens from children. J Clin Virol 2004;31(2):123-129.

77. Lanari M, Giovannini M, Giuffre L, Marini A, Rondini G, Rossi GA, Merolla R, Zuccotti GV, Salvioli GP. Prevalence of respiratory syncytial virus infection in Italian infants hospitalized for acute lower respiratory tract infections, and association between respiratory syncytial virus infection risk factors and disease severity. Pediatr Pulmonol 2002;33(6):458-465.

78. Latzin P, Frey U, Roiha HL, Baldwin DN, Regamey N, Strippoli MP, Zwahlen M, Kuehni CE. Prospectively assessed incidence, severity, and determinants of respiratory symptoms in the first year of life. Pediatr Pulmonol 2007;42(1):41-50.

79. Lazar I, Weibel C, Dziura J, Ferguson D, Landry ML, Kahn JS. Human metapneumovirus and severity of respiratory syncytial virus disease. Emerg Infect Dis 2004;10(7):1318-1320.

80. Leader S, Kohlhase K. Respiratory syncytial virus-coded pediatric hospitalizations, 1997 to 1999. Pediatr Infect Dis J 2002;21(7):629-632.

81. Leder K, Sinclair MI, Mitakakis TZ, Hellard ME, Forbes A. A community-based study of respiratory episodes in Melbourne, Australia. Aust N Z J Public Health 2003;27(4):399-404.

82. Leeder SR, Corkhill R, Irwig LM, Holland WW, Colley JR. Influence of family factors on the incidence of lower respiratory illness during the first year of life. Br J Prev Soc Med 1976;30(4):203-212.

83. 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(3):1213-1219.

84. Lew JF, Glass RI, Petric M, LeBaron CW, Hammond GW, Miller SE, Robinson C, Boutilier J, Riepenhoff-Talty M, Payne CM. Six-year retrospective surveillance of gastroenteritis viruses identified at ten electron microscopy centers in the United States and Canada. Pediatr Infect Dis J 1990;9(10):709-714.

85. López Bravo IM, Sepúlveda H, Valdés I. Acute respiratory illnesses in the first 18 months of life. Rev Panam Salud Publica 1997;1(1):9-17.

86. Macfarlane P, Denham J, Assous J, Hughes C. RSV testing in bronchiolitis: which nasal sampling method is best? Arch Dis Child 2005;90(6):634-635.

87. Mackay IM, Jacob KC, Woolhouse D, Waller K, Syrmis MW, Whiley DM, Siebert DJ, Nissen M, Sloots TP. Molecular assays for detection of human metapneumovirus. J Clin Microbiol 2003;41(1):100-105.

88. Madhi SA, Klugman KP. A role for Streptococcus pneumoniae in virus-associated pneumonia. Nat Med 2004;10(8):811-813.

89. Madhi SA, Ludewick H, Kuwanda L, Niekerk N, Cutland C, Little T, Klugman KP. Pneumococcal coinfection with human metapneumovirus. J Infect Dis 2006;193(9):1236-1243.

90. Maertzdorf J, Wang CK, Brown JB, Quinto JD, Chu M, de Graaf M, van den Hoogen BG, Spaete R, Osterhaus AD, Fouchier RA. Real-time reverse transcriptase PCR assay for detection of human metapneumoviruses from all known genetic lineages. J Clin Microbiol 2004;42(3):981-986.

91. 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(7):2987-2991.

92. Manoha C, Espinosa S, Aho SL, Huet F, Pothier P. Epidemiological and clinical features of hMPV, RSV and RVs infections in young children. J Clin Virol 2007;38(3):221-226.

93. McAdam AJ, Hasenbein ME, Feldman HA, Cole SE, Offermann JT, Riley AM, Lieu TA. Human metapneumovirus in children tested at a tertiary-care hospital. J Infect Dis 2004;190(1):20-26.

94. McIntosh K. Respiratory Syncytial Virus Infections in Infants and Children: Diagnosis and Treatment. Pediatrics in Review 1987;9(6):191-196.

95. Mentel R, Wegner U, Bruns R, Gurtler L. Real-time PCR to improve the diagnosis of respiratory syncytial virus infection. J Med Microbiol 2003;52(Pt 10):893-896.

96. Monto AS, Ullman BM. Acute respiratory illness in an American community. The Tecumseh study. JAMA 1974;227(2):164-169.

97. Mullins JA, Erdman DD, Weinberg GA, Edwards K, Hall CB, Walker FJ, Iwane M, Anderson LJ. Human metapneumovirus infection among children hospitalized with acute respiratory illness. Emerg Infect Dis 2004;10(4):700-705.

98. Murphy BR, Alling DW, Snyder MH, Walsh EE, Prince GA, Chanock RM, Hemming VG, Rodriguez WJ, Kim HW, Graham BS. Effect of age and preexisting antibody on serum antibody response of infants and children to the F and G glycoproteins during respiratory syncytial virus infection. J Clin Microbiol 1986;24(5):894-898.

99. Navas L, Wang E, de Carvalho V, Robinson J. Improved outcome of respiratory syncytial virus infection in a high-risk hospitalized population of Canadian children. Pediatric Investigators Collaborative Network on Infections in Canada. J Pediatr 1992;121(3):348-354.

100. Nickel R, Kulig M, Forster J, Bergmann R, Bauer CP, Lau S, Guggenmoos-Holzmann I, Wahn U. Sensitization to hen's egg at the age of twelve months is predictive for allergic sensitization to common indoor and outdoor allergens at the age of three years. J Allergy Clin Immunol 1997;99(5):613-617.

101. Nielsen AM, Koefoed BG, Møller R, Laursen B. [Prevalence rates of recent illnesses in Danish children, 1994 and 2000] (Danish). Ugeskr Laeger 2006;168(4):373-378.

102. Nielsen AM, Rasmussen S, Christoffersen MN. [Morbidity of Danish infants during their first months of life. Incidence and risk factors] (Danish). Ugeskr Laeger 2002;164(48):5644-5648.

103. Nielsen HE, Siersma V, Andersen S, Gahrn-Hansen B, Mordhorst CH, Nørgaard-Pedersen B, Røder B, Sørensen TL, Temme R, Vestergaard BF. Respiratory syncytial virus infection-risk factors for hospital admission: a case-control study. Acta Paediatr 2003;92(11):1314-1321.

104. Ogra PL. Respiratory syncytial virus: the virus, the disease and the immune response. Paediatr Respir Rev 2004;5 Suppl A:S119-S126.

105. Ordás J, Boga JA, Alvarez-Argüelles M, Villa L, Rodriguez-Dehli C, de Oña M, Rodríguez J, Melón S. Role of metapneumovirus in viral respiratory infections in young children. J Clin Microbiol 2006;44(8):2739-2742.

106. Patel JA, Nguyen DT, Revai K, Chonmaitree T. Role of respiratory syncytial virus in acute otitis media: implications for vaccine development. Vaccine 2007;25(9):1683-1689.

107. Peiris JS, Tang WH, Chan KH, Khong PL, Guan Y, Lau YL, Chiu SS. Children with respiratory disease associated with metapneumovirus in Hong Kong. Emerg Infect Dis 2003;9(6):628-633.

108. Percivalle E, Sarasini A, Visai L, Revello MG, Gerna G. Rapid detection of human metapneumovirus strains in nasopharyngeal aspirates and shell vial cultures by monoclonal antibodies. J Clin Microbiol 2005;43(7):3443-3446.

109. Perry TT, Wood RA, Matsui EC, Curtin-Brosnan J, Rand C, Eggleston PA. Room-specific characteristics of suburban homes as predictors of indoor allergen concentrations. Ann Allergy Asthma Immunol 2006;97(5):628-635.

110. Platt J, Bucknall RA. The disinfection of respiratory syncytial virus by isopropanol and a chlorhexidine-detergent handwash. J Hosp Infect 1985;6(1):89-94.

111. Pullan CR, Hey EN. Wheezing, asthma, and pulmonary dysfunction 10 years after infection with respiratory syncytial virus in infancy. Br Med J (Clin Res Ed) 1982;284(6330):1665-1669.

112. Pullan CR, Toms GL, Martin AJ, Gardner PS, Webb JK, Appleton DR. Breast-feeding and respiratory syncytial virus infection. Br Med J 1980;281(6247):1034-1036.

113. Robinson JL, Lee BE, Bastien N, Li Y. Seasonality and clinical features of human metapneumovirus infection in children in Northern Alberta. J Med Virol 2005;76(1):98-105.

114. Roca A, Quinto L, Saute F, Thompson R, Aponte JJ, Alonso PL. Community incidences of respiratory infections in an actively followed cohort of children <1 year of age in Manhica, a rural area of southern Mozambique. Trop Med Int Health 2006;11(3):373-380.

115. Rohwedder A, Keminer O, Forster J, Schneider K, Schneider E, Werchau H. Detection of respiratory syncytial virus RNA in blood of neonates by polymerase chain reaction. J Med Virol 1998;54(4):320-327.

116. Rossi GA, Medici MC, Arcangeletti MC, Lanari M, Merolla R, Paparatti UD, Silvestri M, Pistorio A, Chezzi C. Risk factors for severe RSV-induced lower respiratory tract infection over four consecutive epidemics. Eur J Pediatr 2007.

117. Ruuskanen O. Respiratory syncytial virus - is it preventable? J Hosp Infect 1995;30 Suppl:494-497.

118. Sandora TJ, Taveras EM, Shih MC, Resnick EA, Lee GM, Ross-Degnan D, Goldmann DA. A randomized, controlled trial of a multifaceted intervention including alcohol-based hand sanitizer and hand-hygiene education to reduce illness transmission in the home. Pediatrics 2005;116(3):587-594.

119. Schauer U, Hoffjan S, Bittscheidt J, Kochling A, Hemmis S, Bongartz S, Stephan V. RSV bronchiolitis and risk of wheeze and allergic sensitisation in the first year of life. Eur Respir J 2002;20(5):1277-1283.

120. Schildgen O, Glatzel T, Geikowski T, Scheibner B, Matz B, Bindl L, Born M, Viazov S, Wilkesmann A, Knopfle G, Roggendorf M, Simon A. Human metapneumovirus RNA in encephalitis patient. Emerg Infect Dis 2005;11(3):467-470.

121. Semple MG, Cowell A, Dove W, Greensill J, McNamara PS, Halfhide C, Shears P, Smyth RL, Hart CA. Dual infection of infants by human metapneumovirus and human respiratory syncytial virus is strongly associated with severe bronchiolitis. J Infect Dis 2005;191(3):382-386.

122. Sherriff A, Peters TJ, Henderson J, Strachan D. Risk factor associations with wheezing patterns in children followed longitudinally from birth to 3(1/2) years. Int J Epidemiol 2001;30(6):1473-1484.

123. Sigurs N, Bjarnason R, Sigurbergsson F, Kjellman B. Respiratory syncytial virus bronchiolitis in infancy is an important risk factor for asthma and allergy at age 7. Am J Respir Crit Care Med 2000;161(5):1501-1507.

124. Sigurs N, Bjarnason R, Sigurbergsson F, Kjellman B, Bjorksten B. Asthma and immunoglobulin E antibodies after respiratory syncytial virus bronchiolitis: a prospective cohort study with matched controls. Pediatrics 1995;95(4):500-505.

125. Sigurs N, Gustafsson PM, Bjarnason R, Lundberg F, Schmidt S, Sigurbergsson F, Kjellman B. Severe respiratory syncytial virus bronchiolitis in infancy and asthma and allergy at age 13. Am J Respir Crit Care Med 2005;171(2):137-141.

126. Simoes EA. Respiratory syncytial virus infection. Lancet 1999;354(9181):847-852.

127. Sloots TP, Mackay IM, Bialasiewicz S, Jacob KC, McQueen E, Harnett GB, Siebert DJ, Masters BI, Young PR, Nissen MD. Human metapneumovirus, Australia, 2001-2004. Emerg Infect Dis 2006;12(8):1263-1266.

128. Staat MA. Respiratory syncytial virus infections in children. Semin Respir Infect 2002;17(1):15-20.

129. 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(9178):541-545.

130. Stensballe LG, Trautner S, Kofoed PE, Nante E, Hedegaard K, Jensen IP, Aaby P. Comparison of nasopharyngeal aspirate and nasal swab specimens for detection of respiratory syncytial virus in different settings in a developing country. Trop Med Int Health 2002;7(4):317-321.

131. Stensballe LG, Kristensen K, Simoes EA, Jensen H, Nielsen J, Benn CS, Aaby P, for the Danish RSV Data Network. Atopic Disposition, Wheezing, and Subsequent Respiratory Syncytial Virus Hospitalization in Danish Children Younger Than 18 Months: A Nested Case-Control Study. Pediatrics 2006;118(5):e1360-e1368.

132. Strangert K. Respiratory illness in preschool children with different forms of day care. Pediatrics 1976;57(2):191-196.

133. Sundhedsstyrelsen. Antallet af rygere i Danmark falder fortsat (Danish). www.sst.dk / September 2007.

134. Trefny P, Stricker T, Baerlocher C, Sennhauser FH. Family history of atopy and clinical course of RSV infection in ambulatory and hospitalized infants. Pediatr Pulmonol 2000;30(4):302-306.

135. Uldall P. Spæd- og småbørns almindelige sygelighed - forekomst og sociale konsekvenser [Acute illness in preschool children] (Danish). Thesis 1986;(Publ. 18).

136. van den Hoogen BG, de Jong JC, Groen J, Kuiken T, de Groot R, Fouchier RA, Osterhaus AD. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. Nat Med 2001;7(6):719-724.

137. van den Hoogen BG, Osterhaus DM, Fouchier RA. Clinical impact and diagnosis of human metapneumovirus infection. Pediatr Infect Dis J 2004;23(1 Suppl):S25-S32.

138. 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(10):1571-1577.

139. van Strien RT, Verhoeff AP, Brunekreef B, van Wijnen JH. Mite antigen in house dust: relationship with different housing characteristics in The Netherlands. Clin Exp Allergy 1994;24(9):843-853.

140. van Woensel JB, Bos AP, Lutter R, Rossen JW, Schuurman R. Absence of human metapneumovirus co-infection in cases of severe respiratory syncytial virus infection. Pediatr Pulmonol 2006;41(9):872-874.

141. van Benten I, Koopman L, Niesters B, Hop W, van Middelkoop B, de Waal L, van Drunen K, Osterhaus AD, Neijens H, Fokkens W. Predominance of rhinovirus in the nose of symptomatic and asymptomatic infants. Pediatr Allergy Immunol 2003;14(5):363-370.

142. Verbrugge LM. Health diaries. Med Care 1980;18(1):73-95.

143. Viazov S, Ratjen F, Scheidhauer R, Fiedler M, Roggendorf M. High prevalence of human metapneumovirus infection in young children and genetic heterogeneity of the viral isolates. J Clin Microbiol 2003;41(7):3043-3045.

144. Vicente D, Cilla G, Montes M, Perez-Yarza EG, Perez-Trallero E. Human bocavirus, a respiratory and enteric virus. Emerg Infect Dis 2007;13(4):636-637.

145. Vyse AJ, Cohen BJ, Ramsay ME. A comparison of oral fluid collection devices for use in the surveillance of virus diseases in children. Public Health 2001;115(3):201-207.

146. Waris M, Heikkinen T, Osterback R, Jartti T, Ruuskanen O. Nasal swabs for detection of respiratory syncytial virus RNA in children. Arch Dis Child 2007.

147. Whiley DM, Syrmis MW, Mackay IM, Sloots TP. Detection of Human Respiratory Syncytial Virus in Respiratory Samples by LightCycler Reverse Transcriptase PCR. J Clin Microbiol 2002;40(12):4418-4422.

148. Wilkesmann A, Schildgen O, Eis-Hubinger AM, Geikowski T, Glatzel T, Lentze MJ, Bode U, Simon A. Human metapneumovirus infections cause similar symptoms and clinical severity as respiratory syncytial virus infections. Eur J Pediatr 2006;165(7):467-475.

149. Williams JV, Harris PA, Tollefson SJ, Halburnt-Rush LL, Pingsterhaus JM, Edwards KM, Wright PF, Crowe JE, Jr. Human metapneumovirus and lower respiratory tract disease in otherwise healthy infants and children. N Engl J Med 2004;350(5):443-450.

150. Xatzipsalti M, Kyrana S, Tsolia M, Psarras S, Bossios A, Laza-Stanca V, Johnston SL, Papadopoulos NG. Rhinovirus viremia in children with respiratory infections. Am J Respir Crit Care Med 2005;172(8):1037-1040.

151. Xepapadaki P, Psarras S, Bossios A, Tsolia M, Gourgiotis D, Liapi-Adamidou G, Constantopoulos AG, Kafetzis D, Papadopoulos NG. Human Metapneumovirus as a causative agent of acute bronchiolitis in infants. J Clin Virol 2004;30(3):267-270.

11. Appendix. Health diary

Name:_____ Study no._____ Next appointment:_____

Symptoms of the child in **January** (Mark every date):

	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
No symptoms																															
General malaise																															
Nasal discharge/runny nose																															
Cough																															
Fever/feels hot																															
Hoarseness																															
Conjunctivitis																															
Rash/eczema																															
Fast breathing																															
Wheezing																															
Diarrhoea (>3 stools/day)																															
Vomiting																															
Reduced appetite																															
Medicine*																															
Doctor's visit[#]																															
Hospital admission[#]																															

*Type of medicine:

[#]Reason for doctor's visit or hospital admission: