VIRAL RESPIRATORY INFECTIONS: DIAGNOSIS AND EPIDEMIOLOGY

Maria Rotzén Östlund

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ABSTRACT

Background. Respiratory viral infections are common causes of human morbidity and mortality in children as well as in adults. Adenovirus, influenza virus, parainfluenza virus and respiratory syncytial virus (RSV) have been recognized for many years. During recent years two main events have influenced both the diagnosis and our knowledge of respiratory virus epidemiology: (1) Five new viruses have been described; (2) the use of molecular methods for the diagnosis of respiratory tract infections has been introduced.

Objectives. The first objective of this thesis was to study the diversity of respiratory virus infections in Stockholm in different patient groups and using different diagnostic methods. The second objective was to study the molecular epidemiology of RSV. The third objective was to evaluate and compare the conventional diagnostic methods for respiratory viruses with new molecular diagnostic methods.

Materials and Methods. In paper I the diagnostic results from 7303 respiratory specimens were analyzed retrospectively. The results were obtained by virus isolation and antigen detection with immunofluorescence. In paper II RSV strains from 234 frozen respiratory specimens were sequenced and genotyped. In paper III a real-time diagnostic platform for 15 respiratory viruses was developed and evaluated based on 585 frozen nasopharyngeal aspirates and after that evaluated based on diagnostic samples. In paper IV 37 infection episodes with multiple viral findings were compared with 94 infection episodes with single findings.

Results. In paper I one or two viruses were found in 43% of the samples, with the highest proportion of positive samples among the oldest patients. RSV was the most common finding among children under five years of age, and influenza was the most common in adults and children over five years old. RSV was only found in 2% of patients over 81 years old. The genotyping of 234 strains in paper II showed that both RSV subgroups A and B were circulating during the 2002–2003 season. The two subgroups were further subdivided into two genotypes each. A comparison with available strains from other parts of the world showed a high degree of similarity. In paper III the diagnostic yield increased from 37% with conventional methods to 57% with the new diagnostic real-time PCR platform. The children with multiple findings were significantly older than those with single findings in the comparison between the two groups in paper IV. There was no difference between the groups regarding hospitalization time, diagnosis, CRP levels, signs on x-ray or oxygen treatment.

Conclusions. By using a real-time PCR platform for viral respiratory tract infections, the diagnostic yield increased compared to virus isolation and antigen detection. Besides older age, no apparent differences between children with multiple findings and single findings were observed, indicating that prolonged sheddings from sequential infections rather than true multiple infections were detected. The low findings of RSV among the elderly indicate either that RSV is not common among the elderly in Sweden or that RSV infection appears before admission to hospital or that more sensitive methods than IF have to be used in the elderly. The high degree of similarity between strains from Sweden and other parts of the world supports the current theories on RSV epidemiology suggesting that local factors, such as local immunity in the population, are more important for the outcome of epidemics than globally circulating strains.
LIST OF PUBLICATIONS

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

I. Maria Rotzén Östlund, Benita Zweygberg Wirgart, Annika Linde and Lena Grillner.  
Respiratory Virus Infections in Stockholm During Seven Seasons: A Retrospective Study of Laboratory Diagnosis.  

II. Maria Rotzén Östlund, Annika Tiveljung Lindell, Sofia Stenler, Hilde M Riedel, Benita Zweygberg Wirgart and Lena Grillner.  
Journal of Medical Virology 2008; 80:159-167.

Journal of Medical Virology, in press

Manuscript
6.2.2 Decreased processing time
6.2.3 Quantification
6.2.4 Sequencing and genotyping

6.3 Aspects of the interpretation of results
6.3.1 Secretion after and before infection
6.3.2 Viral findings in asymptomatic patients
6.3.3 Multiple findings

7 Conclusions
8 Acknowledgments
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>GMK</td>
<td>Green monkey kidney cells</td>
</tr>
<tr>
<td>HCoV</td>
<td>Human coronavirus</td>
</tr>
<tr>
<td>HMPV</td>
<td>Human metapneumovirus</td>
</tr>
<tr>
<td>HRV</td>
<td>Human rhinovirus</td>
</tr>
<tr>
<td>IF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>LRTI</td>
<td>Lower respiratory tract infection</td>
</tr>
<tr>
<td>Ma-104</td>
<td>Fetal Rhesus Monkey kidney cells-104</td>
</tr>
<tr>
<td>Mdck</td>
<td>Madin-Darby canine kidney cells</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NA</td>
<td>Nucleic acid</td>
</tr>
<tr>
<td>NAT</td>
<td>Nucleic acid amplification technique</td>
</tr>
<tr>
<td>NPA</td>
<td>Nasopharyngeal aspirate</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PIV</td>
<td>Parainfluenza virus</td>
</tr>
<tr>
<td>POC</td>
<td>Point of care</td>
</tr>
<tr>
<td>RSV</td>
<td>Respiratory syncytial virus</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SARS</td>
<td>Severe acute respiratory syndrome</td>
</tr>
<tr>
<td>URTI</td>
<td>Upper respiratory tract infection</td>
</tr>
</tbody>
</table>
1 INTRODUCTION

Acute respiratory tract infections are one of the most common causes of morbidity and hospitalization in children and adults. Prospective epidemiological studies have estimated the annual frequency of respiratory disease episodes to be 4-8 for children up to five years of age and 2-4 in adults, and declining with age [Lambert et al., 2007; Monto, 1994]. In developing countries, pneumonia and other respiratory tract infections are still one of the leading causes of mortality in childhood [Bryce et al., 2005].

Before the year 2000, viral respiratory tract infections were considered to be caused mainly by adenovirus, human coronavirus (HCoV) OC43 and 229E, influenza A or B virus, parainfluenza virus (PIV) 1-4, respiratory syncytial virus (RSV), and rhinovirus. Diagnostic methods such as viral isolation and antigen detection methods were widely used for the diagnosis of adenovirus, influenza virus, PIV, and RSV. Methods for diagnosing coronavirus and rhinovirus were not developed for daily use in the hospital laboratory. These viruses were considered rather harmless common cold viruses and therefore not of importance for hospitalized patients.

Since 2001 five new viruses have been described. Four of them, human metapneumovirus (HMPV), the two coronaviruses HCoV-NL63 and HCoV-HKU1, and human bocavirus have probably been circulating among humans for a long time [Allander et al., 2005; Pyrc et al., 2007; van den Hoogen et al., 2001; van der Hock et al., 2004; Woo et al., 2005]. The fifth newly discovered virus, the human coronavirus SARS (SARS-CoV), is assumed to have entered the human population in 2002, causing the global epidemic of severe acute respiratory syndrome (SARS) [Peiris et al., 2003]. After the epidemic in 2002-2003 a few laboratory-acquired SARS cases have occurred, but there are no signs that SARS-CoV still circulates in the human population [Peiris et al., 2004].

The clinical significance of most, but not all, of the former known respiratory viruses is quite well known. However, some important new insights have been pointed out during recent years. One is the importance and impact of RSV among adults and the elderly. To be able to study the frequency of RSV and other viruses among hospitalized patients in a Swedish series, a retrospective study of respiratory viruses was done on results from respiratory samples from six seasons.

The discovery of the new viruses has been made possible by the molecular techniques. These techniques have also altered the diagnostic possibilities for viral respiratory tract infections. A shift from conventional methods such as virus isolation and immunofluorescence (IF) to PCR and other nucleic acid amplification techniques is therefore necessary. In paper I we evaluated virus isolation and antigen detection with IF and in paper III we adapted the molecular techniques to the clinical laboratory in order to give the patients a sensitive and rapid result for all important viral respiratory tract infections.
The frequency of multiple findings in respiratory specimens has increased using molecular diagnostic methods. The clinical impact of multiple findings needs to be further investigated, and the work in paper IV deals with this issue.

Most respiratory viruses are subdivided into subgroups, genotypes, and serotypes. The clinical and epidemiological impact of infections with different subtypes of respiratory viruses needs to be studied further. RSV consists of two subgroups, A and B, and is further subdivided into several genotypes. In order to study circulating strains and compare them with available strains from other parts of the world, all RSV positive samples from one season in Stockholm were genotyped, which is described in paper II.
2 BACKGROUND

2.1 RESPIRATORY VIRUSES

Respiratory viruses are found in the following virus families: *Coronaviridae*, *Orthomyxoviridae*, *Paramyxoviridae*, *Picornaviridae* (RNA viruses), *Adenoviridae* and *Parvoviridae* (DNA viruses) (Table 1).

Viruses from the *Herpesviridae* family such as cytomegalovirus (CMV) and herpes-simplex virus are also known respiratory tract pathogens, but since these viruses mainly produce respiratory symptoms in immunocompromised patients they will not be further discussed in this thesis. Nor will enterovirus, morbillivirus and varicella-zoster virus be further mentioned. Enterovirus is a virus found in the respiratory tract, mainly in infants, but is not considered to be a virus primarily causing respiratory tract infection, but rather a systemic infection engaging the airways. The respiratory symptoms associated with morbillivirus and varicella-zoster virus are clearly bound to measles and chickenpox, respectively.
<table>
<thead>
<tr>
<th>Family</th>
<th>Subfamily</th>
<th>Genus</th>
<th>Species</th>
<th>Nucleic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenoviridae</td>
<td>Mastadenovirus</td>
<td>Adenovirus</td>
<td>Adenovirus A-F</td>
<td>DNA</td>
</tr>
<tr>
<td>Parvoviridae</td>
<td>Parvovirinae</td>
<td>Bocavirus</td>
<td>Human bocavirus</td>
<td>DNA</td>
</tr>
<tr>
<td>Coronaviridae</td>
<td>Coronavirinae</td>
<td>Coronavirus</td>
<td>Human coronavirus 229E</td>
<td>RNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Human coronavirus NL63</td>
<td>RNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Human coronavirus OC43</td>
<td>RNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Human coronavirus HKU1</td>
<td>RNA</td>
</tr>
<tr>
<td>Orthomyxoviridae</td>
<td>Influenza A virus</td>
<td>Influenza A virus</td>
<td>Influenza A virus</td>
<td>RNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Influenza B virus</td>
<td>Influenza B virus</td>
<td>RNA</td>
</tr>
<tr>
<td>Paramyxoviridae</td>
<td>Paramyxovirinae</td>
<td>Respirivirus</td>
<td>Parainfluenzavirus type 1</td>
<td>RNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rubulavirus</td>
<td>Parainfluenzavirus type 2</td>
<td>RNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Parainfluenzavirus type 4</td>
<td>RNA</td>
</tr>
<tr>
<td></td>
<td>Pneumovirinae</td>
<td>Pneumovirus</td>
<td>Respiratory syncytial virus</td>
<td>RNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Metapneumovirus</td>
<td>Human metapneumovirus</td>
<td>RNA</td>
</tr>
<tr>
<td>Picornaviridae</td>
<td>Rhinovirus</td>
<td>Human rhinovirus A-C</td>
<td>Human rhinovirus A-C</td>
<td>RNA</td>
</tr>
</tbody>
</table>
2.1.1 Adenovirus

Adenoviruses are common causes of disease in childhood. This DNA virus, described for the first time in 1953 [Hilleman and Werner, 1954; Huebner et al., 1954; Rowe et al., 1953], causes respiratory, gastrointestinal, and conjunctival infections (Table 1). Adenovirus is a common cause of tonsillitis, prolonged fever and high-grade fever in childhood [Ruuskanen et al., 1985].

To date, more than 50 adenovirus serotypes have been described [Horwitz, 2001; Ishiko et al., 2008]. The most common serotypes causing respiratory illness are serotypes 1–4, 7, 14, and 21 [Horwitz, 2001; Ruuskanen, 1997] The incubation period is somewhat longer than for most other respiratory viruses, about 10 days [Ruuskanen, 1997]. Adenovirus can be shed for months in stool (Table 2) [Fox et al., 1977]. Outbreaks of severe pneumonia in military camps and schools have been described, as well as water-borne outbreaks [Dudding et al., 1973; Martone et al., 1980].

A vaccine against adenovirus types 4 and 7 was successfully used in the US army, but the manufacture of these vaccines ceased in the 1990s. No new adenovirus vaccines have been introduced in the US army [Sivan et al., 2007].

At least two antiviral drugs with in vitro activity against adenovirus have been used in humans, ribavirin and cidofovir. Adenoviral therapy is considered in immunocompromised patients with a high risk for adenovirus-related mortality. So far, treatment with ribavirin has been disappointing. Cidofovir appears to be more active in patients than ribavirin, but it has toxic side effects on renal function. None of these agents are commonly used for the treatment of adenovirus infections [Nichols et al., 2008]
Table 2. Duration of Detection of Shed Virus after Infection in Immunocompetent Persons

<table>
<thead>
<tr>
<th>Virus</th>
<th>With virus isolation and antigen detection</th>
<th>With molecular diagnostic methods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus</td>
<td>5-10 days [Horwitz, 2001]</td>
<td>Not known</td>
</tr>
<tr>
<td>Bocavirus</td>
<td>Not known</td>
<td></td>
</tr>
<tr>
<td>Coronavirus</td>
<td>HCoV-229E: Five days [McIntosh, 1997]</td>
<td>HCoV-NL63 and HCoV-OC43: Up to three weeks [Kaiser et al., 2005]</td>
</tr>
<tr>
<td>Human metapneumovirus</td>
<td>Not known</td>
<td></td>
</tr>
<tr>
<td>Influenza A virus</td>
<td>5-7 days [Carrat et al., 2008; Frank et al., 1981]**</td>
<td>Not known**</td>
</tr>
<tr>
<td>Influenza B virus</td>
<td>5-7 days [Carrat et al., 2008; Frank et al., 1981]**</td>
<td>Not known**</td>
</tr>
<tr>
<td>Parainfluenza virus</td>
<td>Mean 7 -10 days [Chanock et al., 2001]</td>
<td>Not known</td>
</tr>
<tr>
<td>Rhinovirus</td>
<td>Up to three weeks [Couch, 2001]</td>
<td>One to six weeks [Jartti et al., 2004; Winther et al., 2006]</td>
</tr>
<tr>
<td>Respiratory syncytial virus</td>
<td>Infants: mean 6.7 days (range 1-21) [Hall et al., 1976]</td>
<td>Not known</td>
</tr>
</tbody>
</table>

* Weeks to months in stool [Fox et al., 1977]
** PCR methods included in the review by Carrat

2.1.2 Coronavirus

Human coronaviruses have been considered to be harmless common cold viruses ever since they were described for the first time in the 1960s [Hamre and Procknow, 1966; Tyrrell and Bynoe, 1965]. In 2002 and 2003 the world was struck by the global epidemic of the severe acute respiratory illness, SARS. An international effort of the scientific society showed that SARS was caused by a former unknown coronavirus, SARS-CoV [Peiris et al., 2003]. The SARS epidemic declined and, apart from three incidents of laboratory-acquired SARS, the virus seems to have disappeared from the human population [Peiris et al., 2004]. After the SARS epidemic, the interest in the coronavirus increased and since 2003 two other new coronaviruses have been described [van der Hoek et al., 2004; Woo et al., 2005]. Human coronaviruses are divided into two groups, coronavirus groups I and II (Table 3).
Table 3. Human coronaviruses

<table>
<thead>
<tr>
<th>Group</th>
<th>Species</th>
<th>Year of discovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>HCoV-229E</td>
<td>1960s</td>
</tr>
<tr>
<td></td>
<td>HCoV-NL63</td>
<td>2004</td>
</tr>
<tr>
<td>Group II</td>
<td>HCoV-OC43</td>
<td>1960s</td>
</tr>
<tr>
<td></td>
<td>HCoV-HKU1</td>
<td>2005</td>
</tr>
<tr>
<td></td>
<td>SARS-CoV</td>
<td>2003</td>
</tr>
</tbody>
</table>

2.1.2.1 HCoV-OC43 and HCoV-229E

The first coronaviruses were discovered in the 1960s. At least two serologically distinct viruses were then characterized, HCoV-OC43 and HCoV-229E [Hamre and Procknow, 1966; Tyrrell and Bynoe, 1965]. Other coronaviruses were also investigated at that time, but unfortunately the strains were lost and they are no longer available for follow-up studies [van der Hoek, 2007].

The two coronavirus strains HCoV-OC43 and HCOV-229E have been inoculated in healthy adult volunteers (“handkerchief studies”) and proved to be clearly associated with the common cold [Bradburne et al., 1967]. HCoV-OC43 and HCoV-229E are the second most common cause of the common cold [Mäkela et al., 1998]. Epidemiologic studies have also associated other upper and lower respiratory tract illness with HCoV-229E and HCoV-OC43 in children [van Elden et al., 2004].

HCoV-OC43 and HCOV-229E are transmitted and detected most frequently during the winter season in temperate climate countries [Hendley et al., 1972].

2.1.2.2 HCoV-NL63

HCoV-NL63 was first described by a group from the Netherlands in 2004 [van der Hoek et al., 2004]. At nearly the same time, two other groups described new coronaviruses, but later on these three coronaviruses were found to be the same virus, called HCoV-NL63 [Esper et al., 2005b; Fouchier et al., 2004]. This virus is clearly associated with croup, as clarified in a study with a control group [van der Hoek et al., 2005]. It is also associated with other symptoms of upper and lower respiratory tract infection [van der Hoek, 2007]. The virus is ubiquitous and serological studies in adults have shown that seropositivity is common [Hofmann et al., 2005; van der Hoek et al., 2006]. The seasonality of HCoV-NL63 in temperate climate countries seems to be concentrated on the winter season, the same as with HCoV-OC43 and HCoV-229E [van der Hoek et al., 2006].

Early studies directly after the first characterization found an association with Kawasaki disease, but later studies have not confirmed this association and, for the time being, HCoV-NL63 is not considered to be the cause of Kawasaki disease [Dominguez et al., 2006; Esper et al., 2005a; van der Hoek, 2007].
2.1.2.3 HCoV-HKU1
Another coronavirus, HCoV-HKU1, was described in Hong Kong in 2005 and is also associated with respiratory symptoms [Woo et al., 2005]. In the available studies, this virus appears to be ubiquitous but less frequent than other coronaviruses [Esper et al., 2006; Sloots et al., 2006]. Serological studies have shown that only 2% of healthy adults have a significant antibody titer [Woo et al., 2005]. The epidemiological and clinical significance need to be further investigated.

2.1.3 Paramyxovirus
The family *Paramyxoviridae* is divided into two subfamilies, *Paramyxovirinae* and *Pneumovirinae*. The subfamilies are further divided into four and two genera, respectively (Table 1) [Chanock et al., 2001].

2.1.3.1 Respiratory syncytial virus (RSV)
RSV infection is the most common cause of lower respiratory tract infection among children under two years of age. This pneumovirus was first described in 1956 [Blount et al., 1956] and was detected in a chimpanzee suffering from the common cold and was later on isolated in infants [Chanock and Finberg, 1957; Chanock et al., 1957].

RSV is a negative-stranded RNA virus. There are two subgroups, A and B. Subgroup A is further divided into nine genotypes, GA1–GA7, SAA1, and JaA1 and subgroup B into nine genotypes, GB1–GB4, SAB1–SAB3, JaB1 and BA [Kuroiwa et al., 2005; Peret et al., 2000; Peret et al., 1998; Sato et al., 2005; Venter et al., 2001]. The two subgroups often cocirculate during one season, mostly with one subgroup dominating [Sullender, 2000]. Although a possible connection between subgroup and disease severity has not been established, this possibility still needs to be thoroughly investigated with regard to the different genotypes [Fodha et al., 2007].

The RSV genome codes for at least 11 proteins [Cane, 2001]. The attachment (G) glycoprotein is, together with the fusion protein (F), an important antigen that stimulates the immune response [Cane, 2001; Sullender, 2000] (table 4). The G protein coding gene is one of the most genetically variable parts of the genome. This gene consists of two variable parts, the N-terminal and the C-terminal [Cane, 2001; Cane et al., 1991; Johnson et al., 1987; Sullender, 2000; Sullender et al., 1991].

Early studies indicated that RSV was a ubiquitous virus, and then there was soon serological evidence that nearly all children have been infected during their first two years of life [Glezen et al., 1986].

The incubation period is estimated to be 4–5 days [Collins et al., 2001]. RSV is transmitted through large droplets and not by aerosol [Hall and Douglas, 1981]. Early studies of shedding showed that RSV could be shed for 6.7 days (mean) with a range of 1–21 days [Hall et al., 1976].
Table 4. RSV Genes, Proteins and Functions of Proteins

<table>
<thead>
<tr>
<th>Gene</th>
<th>Proteins</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Structural proteins</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>- Surface glycoproteins</strong></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>Fusion (F)</td>
<td>Virus penetration. Major antigen</td>
</tr>
<tr>
<td>G</td>
<td>Attachment (G)</td>
<td>Virus attachment. Major antigen</td>
</tr>
<tr>
<td>SH</td>
<td>Small hydrophobic (SH)</td>
<td>Function unknown. May be non-structural.</td>
</tr>
<tr>
<td></td>
<td><strong>- Matrix proteins</strong></td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>Matrix (M1)</td>
<td>May mediate attachment between nucleocapsid and envelope</td>
</tr>
<tr>
<td></td>
<td><strong>- Nucleocapsid-associated proteins</strong></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>Nucleoprotein (N)</td>
<td>Major RNA-binding nucleocapsid protein.</td>
</tr>
<tr>
<td>P</td>
<td>Phosphoprotein (P)</td>
<td>Polymerase cofactor.</td>
</tr>
<tr>
<td>L</td>
<td>Large polymerase complex (L)</td>
<td>RNA-polymerase</td>
</tr>
<tr>
<td>M2</td>
<td>Small envelope (M2-1 or 22K)</td>
<td>Transcription elongating factor.</td>
</tr>
<tr>
<td></td>
<td>Small envelope (M2-2)</td>
<td>Regulation of transcription</td>
</tr>
<tr>
<td></td>
<td><strong>- Nonstructural proteins</strong></td>
<td></td>
</tr>
<tr>
<td>NS1</td>
<td>Nonstructural protein (NS1)</td>
<td>Function unknown. Involved in response to antiviral interferon activity?</td>
</tr>
<tr>
<td>NS2</td>
<td>Nonstructural protein (NS2)</td>
<td></td>
</tr>
</tbody>
</table>

Modified after Cane, Collins and Hall [Cane, 2001; Collins et al., 2001; Hall, 2001].
The most severe manifestation of RSV in young children is bronchiolitis. 0.8-1.4 % of RSV-infected previously healthy children in Sweden require hospitalization [Eriksson et al., 2002]. Reinfections, with symptoms such as the common cold, are observed at all ages [Glezen et al., 1986]. In the elderly, an RSV infection can cause a severe respiratory tract infection. [Dowell et al., 1996; Falsey et al., 2005; Falsey and Walsh, 2000]. RSV appears every year in epidemics, and in Stockholm, as well as in many other geographic areas, a biennial pattern with alternating minor and major epidemics is seen [Eriksson et al., 2002; Mlinaric-Galinovic et al., 2008; Waris, 1991].

The effort to compose a vaccine against RSV started in the 1960s, but the trials failed at an early stage. Severe disease was observed in some of the vaccinated children and two children died. There is still no available vaccine against RSV [Girard et al., 2005; Power, 2008]. Children at risk can undergo prophylactic treatment with a humanized antibody, palivizumab, during the RSV season [Swedish-concensus-group, 2001; Venkatesh and Weisman, 2006] (www.lakemedelsverket.se). There is no effective antiviral treatment either. Ribavirin has been used in severe cases, but two systematic reviews of randomized trials have concluded that there is no evidence of effectively treating lower respiratory tract infections caused by RSV with ribavirin [King et al., 2004; Randolph and Wang, 1996].

2.1.3.2 Parainfluenza virus
Parainfluenza virus types 1 and 3 belong to the genus Respirovirus of the Paramyxovirinae subfamily, and parainfluenza virus types 2 and 4 to the genus Rubulavirus of the same subfamily (Table 1). These viruses were first described in the late 1950s [Andrewes et al., 1959; Chanock, 1956; Chanock et al., 1958a; Chanock et al., 1958b]. Parainfluenza viruses have a negative-stranded RNA genome. Subtypes of PIV 1 and 3-4 have been described [Henrickson, 2003].

PIV subtypes 1-3 are known to cause both upper and lower respiratory tract infections, including croup [Henrickson, 2003]. PIV 1 is mostly associated with croup, and the peak incidence of infection is during the second and third years. LRTI due to PIV 2 is less frequently reported than PIV 1 and 3. Primary infection with PIV 2 is most likely to occur in the second year of life. PIV 3 is a common cause of bronchiolitis and pneumonia in early childhood, with a peak incidence in the first year of life. PIV 1 and 2 usually appear in the fall and winter, and PIV 3 in the spring and summer [Hall, 2001; Henrickson, 2003]. The importance of PIV 4 is still unclear, although early serological studies showed that most adults present serological evidence of past infection with PIV 4 [Gardner, 1969]. Severe infections with PIV 4 are rarely reported. The immunity to PIV is poor after infection, and reinfections are common [Henrickson, 2003].

The incubation period is 2-8 days and virus is shed for about a week (Table 2). The transmission route is similar to that of RSV [Chanock et al., 2001].

No specific antiviral treatment or vaccine is available for the parainfluenza virus.
2.1.3.3 Human metapneumovirus

Human metapneumovirus (HMPV) was described for the first time in the Netherlands in 2001 [van den Hoogen et al., 2001]. HMPV is a negative-stranded RNA virus. There are two major groups of HMPV, A and B, and each group is subdivided into two more groups, A1-A2 and B1-B2. The denomination of the groups has not yet been decided. Serological studies have found that nearly all children have been infected with HMPV by the age of five [Leung et al., 2005; van den Hoogen et al., 2001].

Human metapneumoviurs is a ubiquitous virus [Dollner et al., 2004; Koetz et al., 2006; Maggi et al., 2003; McAdam et al., 2004; Nicholson et al., 2006; Pelinck et al., 2006; Rafiefard et al., 2008]. The percentage of findings ranges from 5% to 15% in most studies, with some exceptions for a higher detection rate [Kahn, 2006]. HMPV occurs in epidemics, like most other respiratory viruses in temperate climate countries, during the winter period [Kahn, 2006].

HMPV causes both upper and lower tract infections, similar to those of RSV [van den Hoogen et al., 2004]. No specific manifestation of respiratory tract disease is associated with HMPV [Kahn, 2006]. Neither a vaccine nor antiviral treatment is available for HMPV.

2.1.4 Orthomyxovirus

Human influenza virus was recovered for the first time in 1933 [Smith et al., 1933], but influenza epidemics have been described earlier in history, for instance, the Russian flu in the 1880s and 1890s. Influenza virus types A and B are known to be important pathogens among adults and the elderly, with a yearly excess mortality among the elderly [Elliot and Fleming, 2008; Reichert et al., 2004].

The genetic variability of influenza A virus arises in two ways. The first is minor changes in the nucleic acid, antigenic drifts, caused by the inability of the virus to proofread and repair errors in replication. The second is major changes, antigenic shifts, occurring when two influenza viruses are present together in a cell. The shift occurs through assortment of the segmented RNA and can, for instance, result in a novel combination of the two important proteins hemagglutinin and neuraminidase. A new influenza virus strain in the human population may cause an epidemic or even a pandemic. Pigs are believed to serve as “mixing vessels” for reassortment, as they can be infected by both swine influenza and human influenza. It has been discussed whether humans infected with both avian influenza and human influenza could also serve as a mixing vessels [De Jong et al., 2000].

There are several types of avian influenza in both wild birds and poultry. The spread of the highly pathogenic avian influenza H5N1 among humans in Hong Kong in 1997 was terminated by slaughtering all poultry in Hong Kong. Unfortunately, the virus reemerged and has been spreading among birds and humans in Asia, Africa and Europe. Up to September 2008, WHO has confirmed 387 human cases and 245 deaths from H5N1 infection (www.who.org).
A vaccine against influenza has been available since the 1940s [Hilleman, 2000]. The vaccine consists at present of two influenza A and one influenza B strains and is redesigned every six months by an expert committee from the WHO (www.who.org). In Sweden, the National Board of Health and Welfare recommends that the vaccine should be provided to specific risk groups every year: persons over 65 years old and patients with heart and lung diseases (www.socialstyrelsen.se). In other countries children are also included in the recommendations for vaccination. Two neuraminidase inhibitors, zanamivir and oseltamivir, are available for treatment. Oseltamivir can also be used for prophylaxis [De Clercq, 2006] (www.lakemedelsverket.se).

2.1.4.1 Influenza A
Influenza virus A is a negative-stranded, segmented RNA virus, with a broad host range (humans, swine, birds, ferrets, etc.). It appears in epidemics of varying magnitude every year.

A typical influenza illness starts with a rapid onset of fever, malaise, muscle pain, and cough. It lasts at least a week with a convalescent period of a second week. Influenza A appears in all age groups. Bacterial pneumonia is a common complication in the elderly [Treanor, 2000].

2.1.4.2 Influenza B
Influenza virus B is also a negative-stranded, segmented RNA virus. The host range is narrower than for influenza A. This is the main reason why antigen shifts and reassortment do not occur in influenza B [Chen and Holmes, 2008].

Influenza B usually occurs in epidemics with a lower prevalence than influenza A. The illness is quite similar to infection with influenza A, but it is usually of less severity. Some differences are noted in the clinical presentation; for instance, gastrointestinal symptoms are more common in influenza B virus infections [Wright and Webster, 2001].

2.1.5 Picornavirus

2.1.5.1 Rhinovirus
Infection with rhinovirus is the most common cause of respiratory tract illness worldwide [Mackay, 2008; Rotbart and Hayden, 2000]. This negative-stranded RNA virus, known since 1956, has been subdivided into two species, human rhinovirus species A and B (HRV A-B), and each species have been further subdivided into several serotypes [Couch, 2001; Price, 1956]. During recent years a new species, human rhinovirus C (HRV C), has been described [Kistler et al., 2007; Lau et al., 2007; Mackay, 2008]. This new rhinovirus species, HRV-C, has been associated with more severe symptoms than with rhinoviruses species described earlier, although this association needs to be studied further [Mackay, 2008].

The common cold is clearly associated with rhinovirus, as shown in early volunteer studies [Taylor-Robinson and Bynoe, 1964]. More than half of all common colds are caused by rhinovirus [Mäkela et al., 1998]. Immunity after infection is poor, and reinfections are common [Mackay, 2008]. Besides the widespread impact of the common cold, the rhinovirus
is the most frequent cause of exacerbations of wheezing among adults and older children [Gern, 2002; Johnston et al., 1995; Mackay, 2008; Nicholson et al., 1993; Traves and Proud, 2007].

Rhinovirus infections occur during the whole year, but they are most frequent in the spring and fall [Mackay, 2008; Winther et al., 2006].

There is no vaccine or antiviral treatment for rhinovirus, although there have been several potential candidates for antiviral treatment [Mackay, 2008; Nichols et al., 2008].

2.1.6 Parvovirus

2.1.6.1 Bocavirus

Human bocavirus, a DNA virus belonging to the Parvoviridae family, was described in Sweden for the first time in 2005 [Allander et al., 2005]. The virus was identified by “molecular virus screening”. This procedure is based on DNase treatment of a large number of samples, followed by random amplification and cloning and, after that, large-scale sequencing and bioinformatic analyses. The same, or nearly the same, technique has led since then also to the identification of KI and WU polyomaviruses [Allander, 2008]. The human bocavirus is the second known human pathogen in the parvovirus family. Serological studies have shown that most children have been infected with bocavirus by the age of five [Kantola et al., 2008].

Bocavirus has been detected in respiratory samples as well as in blood. Acute primary infection is clearly associated with wheezing, but other acute respiratory tract symptoms have also been reported [Allander et al., 2007; Jartti et al., 2007]. The virus has also been frequently detected in multiple respiratory viral findings, most likely reflecting post-infectious shedding and is in this context probably a harmless passenger [Schildgen et al., 2008]. High viral loads and viremia have been found to be associated with primary infections and is followed by seroconversion [Kantola et al., 2008]. Bocavirus has been found in studies all over the world [Allander, 2008]. No seasonal pattern has been described so far [Schildgen et al., 2008].
2.2 DIAGNOSTIC METHODS

Virus isolation and antigen detection by immunofluorescence have been the methods used for decades for the diagnosis of respiratory virus infections. Up to now, virus isolation has been regarded as the golden standard for the diagnosis of adenovirus, influenza virus, PIV, and RSV. More rapid methods such as immunochromatography and enzyme-linked immunoassays have also been in frequent use, especially as point-of-care (POC) tests. Molecular methods such as PCR are now becoming more and more common in the daily diagnostic work flow.

2.2.1 Virus isolation

Virus isolation in cell lines has been used since the 1940s for detection of viruses [Enders et al., 1949]. The advantages of virus isolation are the high sensitivity for several viruses and the possibility of finding other viruses than the expected ones when using a variety of cell lines. Virus isolation is also quite independent of genetic variability in the viral genome, even though a specific cell line could lose the capability of propagating a certain virus.

Virus isolation has some disadvantages. The technique for cell culturing requires trained personnel and special equipment and can therefore only be performed in special laboratories. The inoculated cell lines have to be observed for at least 10–14 days in order to detect most respiratory viruses. The time from sampling to the result will consequently be too long to be of any practical importance for the treatment of the patient. The newly described viruses, as well as coronavirus and rhinovirus, are difficult or impossible to isolate or require special conditions to be propagated. A viable virus is needed for virus isolation, and the sensitivity is therefore lower for vulnerable viruses such as RSV than for other respiratory viruses [Collins et al., 2001].

2.2.2 Antigen detection

Antigen detection with monoclonal antibodies and immunofluorescence (IF) staining is a widely used method for rapid detection of respiratory viruses. The sampling technique is extremely important. A sufficient amount of epithelial cells in the respiratory specimens is required in order to make a proper examination. Trained personnel for examining the slides are also necessary. Antigen detection with IF is fairly inexpensive [Madeley and Peiris, 2002].

IF has a high sensitivity and specificity for especially RSV and influenza A. The sensitivity for influenza B, parainfluenza virus and adenovirus is usually lower. The range of available monoclonal antibodies for IF is limited. For influenza, RSV, parainfluenza virus and adenovirus, there are several commercial antibodies on the market, but validated monoclonal antibodies with high specificity and sensitivity are missing for other respiratory viruses such as rhinovirus, coronavirus, and HMPV.

Immunochromatography and enzyme-linked immunoassays mostly have a lower sensitivity and specificity than IF [Hurt et al., 2007; Smit et al., 2007]. These tests are rapid and easy to
perform and interpret. They are therefore suitable for use in highly epidemic situations and as a complement to more sensitive methods during emergency hours [Nilsson et al., 2008].

### 2.2.3 Nucleic acid amplification techniques

Nucleic acid amplification techniques (NATs) are becoming more widely used and are the predominant diagnostic method for respiratory viruses in many laboratories. The range of applications for PCR has broadened since its first introduction in the laboratory. The early PCR methods using manual NA extraction, nested PCR, and detection with gel-electrophoresis were time-consuming. With the introduction of real-time PCR and automated NA extraction, the time to result has shortened.

The diagnosis of the newly discovered viruses, HMPV, HCoV-NL63, HCoV-HKU1, and bocavirus relies only on the use of molecular diagnostic methods. This has stressed the development of fast workflows, automation, and instrumentation which allow a rapid molecular diagnosis [Fox, 2007; Gunson et al., 2006].

A drawback of PCR using specific primers and probes is that small genetic drifts in the virus genome may give false negative results due to a lack of binding sites for the primers and probes. A thorough surveillance of circulating strains is therefore necessary in order to have sensitive methods.

### 2.2.4 Serology

Comparing the IgG levels in an acute and a convalescent serum is a sensitive method for diagnosing many respiratory viruses. Serology is useful in prospective studies and has been widely used in early epidemiological studies [Monto, 1994]. In clinical practice, serology is difficult to use. A convalescent serum is drawn not earlier than 10 days after the first day of infection and is then analyzed together with the acute serum. The time to result is therefore too long to be of any practical importance for the care of patients. The IgM response to specific viruses during the acute respiratory illness is limited and of no value for diagnosis. Bocavirus may be one exception [Kantola et al., 2008].

### 2.2.5 Genotyping and sequencing

Amplification of a nucleic acid fragment followed by sequencing is the most frequently used method for genotyping respiratory viruses. It is time-consuming and expensive and usually is not needed in the diagnosis for a specific patient. Genotyping is, however, necessary in the surveillance of respiratory virus infections. Circulating strains from different parts of the world have to be thoroughly compared in order to follow and predict coming epidemics. This is especially true of influenza, but it could be of importance also for other viruses such as RSV.
2.2.6 Phylogenetic analysis
The genetic relationship of different viruses or different strains of a specific virus can be studied by phylogenetic analysis. There are several different methods available for the construction of phylogenetic trees. Phylogenetic analysis is a tool for describing the molecular epidemiology of a certain virus from different geographic areas as well as from different periods of time.
3 AIMS

- To study the diversity and epidemiology of causative viruses of respiratory infections in Stockholm.

- To study the molecular epidemiology of RSV during one season in Stockholm and compare the Stockholm strains with strains from other parts of the world.

- To evaluate antigen detection, virus isolation, and PCR for the diagnosis of respiratory infections.

- To develop a real-time PCR diagnostic platform for respiratory viruses with a streamlined workflow and sustained or improved sensitivity compared to virus isolation and antigen detection.

- To study the clinical impact of multiple respiratory viral findings in children.
4 MATERIAL AND METHODS

4.1 SAMPLING
The majority of samples in this study were nasopharyngeal aspirates (NPAs) collected by using a baby-feeding tube and an aspiration trap [Shen et al., 1996]. After suction, the tube was rinsed with approximately 2 mL of sterile saline solution. Other less common samples were bronchoalveolar lavages (BALs) and tracheal and bronchial aspirates, and nasopharyngeal and throat swabs.
4.2 CLINICAL SPECIMENS

4.2.1 Paper I
The assay results for all respiratory samples analyzed at the Department of Clinical Microbiology, Karolinska University Laboratory, between January 1993 and June 2000 were included in this retrospective study. Altogether, the results from 7303 samples were obtained: 86% NPA, 5% nasopharyngeal swabs, 7% throat swabs, and 2% tracheal and bronchial aspirates or lavages (Table 5).

4.2.2 Paper II
All respiratory samples found to be positive for RSV at the laboratory by either IF and/or virus isolation during the season 2002-2003 were included in this retrospective study. In total, 244 samples were positive for RSV, the majority of which, 241 samples, were NPAs, and only two were BALs and one a throat swab. 234 of the 244 samples could be further analyzed by genotyping. The 234 samples were derived from 216 patients, 61% males and 39% females. The majority of the samples were from children less than one year of age, 79%, and only 3% were from patients over 50 years of age (Table 5).

4.2.3 Paper III

Specimens used in the evaluation of the real-time PCR diagnostic panel

Nasopharyngeal aspirates collected at the Astrid Lindgren Children’s Hospital (Karolinska University Hospital) were used in the evaluation of a new diagnostic platform. 585 consecutive samples from 517 hospitalized patients from July 2004 to June 2005 were included. The majority, 61.4%, of the samples were from children under one year of age (Table 5).

Diagnostic results 2007-2008

Since the operational start of the PCR diagnostic platform in the Department of Clinical Microbiology, Karolinska University Laboratory, in October 2007 up to March 2008, 1322 respiratory samples were analyzed, 1129 NPAs and 193 BALs (Table 5).

4.2.4 Paper IV
Thirty-seven respiratory specimens from 37 patients with multiple infections were chosen from those in paper III. Ninety-four samples from 92 patients from paper III were used as controls. Patient data were then collected from the records (Table 5).
Table 5. Respiratory Samples Used in the Different Papers. All Samples Are from the Department of Clinical Microbiology, Karolinska University Laboratory.

<table>
<thead>
<tr>
<th>Table 5.</th>
<th>Included samples</th>
<th>Number of samples</th>
<th>Time period</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paper I</td>
<td>All</td>
<td>7303</td>
<td>July 1993-June 2000</td>
</tr>
<tr>
<td>Paper II</td>
<td>All RSV-positive samples</td>
<td>244</td>
<td>July 2002-June 2003</td>
</tr>
<tr>
<td>Paper III Evaluation study</td>
<td>All samples from Astrid Lindgrens Children’s hospital</td>
<td>585</td>
<td>July 2004-June 2005</td>
</tr>
<tr>
<td>Paper IV</td>
<td>Multiple findings and single findings as controls from paper III.</td>
<td>131 (37 multiple findings and 94 single findings)</td>
<td>July 2004-June 2005</td>
</tr>
</tbody>
</table>
4.3 METHODS
For details, see papers I-IV.

Table 6. Diagnostic Methods Used in the Different Papers

<table>
<thead>
<tr>
<th>Method</th>
<th>Paper I</th>
<th>Paper II</th>
<th>Paper III*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus isolation</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Antigen detection with IF</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>NAT, PCR, detection with gel electrophoresis</td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAT, Real-time PCR, detection with SYBRgreen</td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAT, Real-time PCR, detection with probe</td>
<td></td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>Sequencing</td>
<td></td>
<td></td>
<td>x</td>
</tr>
</tbody>
</table>

*Paper IV is based on the results from paper III.

4.3.1 Virus isolation (papers I-III)
Virus isolation was performed on all respiratory samples in papers I-III (Table 6). After centrifugation, supernatants were used for virus isolation and were inoculated on at least 3 cell lines and, during the expected influenza season (October–May), on 4 cell lines (Table 7). The inoculated samples were incubated in roller drums at 37°C. Rhinovirus is preferentially isolated at 33°C, and therefore rhinoviruses were not detected.

Table 7. Cell lines used for respiratory viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Cell line</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus</td>
<td>HeLa</td>
</tr>
<tr>
<td>Human metapneumovirus</td>
<td>Ma 104</td>
</tr>
<tr>
<td>Influenza virus</td>
<td>Mdck, Ma 104</td>
</tr>
<tr>
<td>Parainfluenza virus</td>
<td>Ma 104</td>
</tr>
<tr>
<td>Respiratory syncytial virus</td>
<td>HeLa, Ma 104</td>
</tr>
</tbody>
</table>
4.3.2 Antigen detection with IF (paper I-III)
After centrifugation and washing of the remaining pellet, the cells were spotted on multiwell slides and then stained with monoclonal antibodies (Table 6). In order to optimize the sensitivity for influenza, wells of 8 mm in diameter, instead of 4 mm, have been used since 1999.

4.3.3 NAT (papers II-III)
PCR methods for 15 viruses were evaluated in paper III (Table 6). A PCR method for parainfluenza was not included in the evaluation study but was evaluated later on and included in the final diagnostic panel.

4.3.3.1 Nucleic acid extraction
All RNA and DNA material was extracted in Biorobot M48. In paper II, RNA was extracted using the MagAttract Viral RNA kit and, in paper III, DNA and RNA were extracted using the MagAttract Virus Mini M48 kit.

Before deciding to use the MagAttract Virus Mini M48 kit in paper III and further on in the diagnostic platform, the sensitivity using the total NA kit, as compared to the NA kit extracting DNA or RNA exclusively was evaluated. This pilot study showed that the analytical sensitivity of the two extraction kits was comparable (Table 8).

Table 8. Comparison of Ct values for RSV PCR using an RNA kit and a total NA kit, respectively, for RNA extraction.

<table>
<thead>
<tr>
<th>Sample</th>
<th>MagAttract Viral RNA kit</th>
<th>MagAttract Virus Mini M48 kit</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>26.94</td>
<td>26.74</td>
</tr>
<tr>
<td>2</td>
<td>25.19</td>
<td>24.28</td>
</tr>
<tr>
<td>3</td>
<td>20.36</td>
<td>20.55</td>
</tr>
<tr>
<td>4</td>
<td>26.75</td>
<td>27.79</td>
</tr>
<tr>
<td>5</td>
<td>26.49</td>
<td>27.85</td>
</tr>
<tr>
<td>6</td>
<td>24.69</td>
<td>25.91</td>
</tr>
<tr>
<td>7</td>
<td>23.50</td>
<td>23.42</td>
</tr>
<tr>
<td>8</td>
<td>25.75</td>
<td>26.85</td>
</tr>
<tr>
<td>9</td>
<td>26.99</td>
<td>27.99</td>
</tr>
<tr>
<td>10</td>
<td>26.98</td>
<td>28.81</td>
</tr>
<tr>
<td>11</td>
<td>30.29</td>
<td>30.91</td>
</tr>
<tr>
<td>12</td>
<td>28.92</td>
<td>28.51</td>
</tr>
</tbody>
</table>
4.3.3.2 Primers
The primers for the phylogenetic analysis of RSV (paper II) were designed to include the variable N-terminal region of the G gene in order to be able to compare the results with those of another European study [Zambon et al., 2001]. After concluding our study, the knowledge gained about the genetic variability of the C-terminal led to the classification of RSV subgroup A genotypes based on the C-terminal and not the N-terminal.

New primers in paper III were designed to have optimal sensitivity and specificity for the diagnosis of respiratory tract disease. Some primers have been described previously in the literature.

4.3.3.3 Probes (paper III-IV)
Different types of probes were used in paper III. In the evaluation study the majority of the probes were based on the Taqman technology (hydrolysis probes) with different dyes. One probe (HMPV) was a minor groove binding probe (MGB). Picornaviruses were detected by a SYBR green assay and melting curve analysis and optimized for rhinovirus. In the final diagnostic platform, this assay was replaced by two assays: an enterovirus assay and a rhinovirus assay, both using hydrolysis probes. The advantage was to optimize the sensitivity as well as to make it possible to diagnose both rhinovirus and enterovirus. The rhinovirus and enterovirus assays are optimized for each virus species but some cross-reactivity occurs because of a close sequence similarity.

4.3.3.4 Reverse transcriptase PCR
Since most respiratory viruses are RNA viruses, a reverse transcriptase PCR (RT-PCR) assay is needed to get a complementary DNA (cDNA). There are two options: a one-step PCR involving both the reverse transcriptase reaction and the amplification of the target in one tube, or a two-step procedure with the reverse transcriptase reaction and the PCR reaction separated. The two-step RT-PCR would increase the time required for the assay. Few studies comparing the diagnostic sensitivity between these two procedures have been published [Wacker and Godard, 2005]. In order to have a fast and streamlined procedure, we chose to evaluate the one-step procedure for the diagnostic platform. The results obtained for our assays in quality assurance panels from Quality Control for Molecular Diagnostics (QCMD) have been satisfying using the one-step procedure.

4.3.3.5 Sequencing and phylogenetic analyses (paper II)
The sequencing and subsequent phylogenetic analyses and genotyping were based on a fragment of 345 nucleotides from the N-terminal part of the G gene. The strains from Stockholm were compared to reference strains from the GenBank database. The classification of the reference strains is based on the phylogeny data of the C-terminal part of the G-protein coding gene.
4.3.4 Clinical data (papers I-IV)
Only basic demographic data such as age and gender were recorded for the patients providing the samples in papers I-III.

Clinical data on 129 patients were obtained retrospectively in paper IV. The data were collected retrospectively by reviewing of the medical records by a pediatrician.

4.3.5 Statistical methods (paper IV)
Categorical data were examined using the $\chi^2$ test. Mann-Whitney tests were used to compare continuous data between two groups, and they were performed with the SPSS and the Statistica software.
5 RESULTS

5.1 PAPER I

In this retrospective analysis of the diversity of viral findings in respiratory tract infections in Stockholm, the results and basic demographic data from 7303 samples were obtained from the laboratory information system. The majority of samples, 93%, were collected from patients attending hospital. 56% were samples from patients under 5 years of age, 20% from adults between 21 and 60 years old and 18% from patients older than 60.

A virus was found in 43% of the samples, and two viruses were found in 0.06%. The most common finding was RSV, found in 18% of the samples, followed by influenza A (16%), parainfluenza 1-3 (3%), influenza B (3%), and adenovirus (2%). Enterovirus, herpes simplex virus, and measles were also found in the respiratory specimens in 19 (<1%), 76 (1%), and 12 (<1%) samples, respectively.

Among young children under five years of age, RSV was the most common finding: 79% of the viral findings in children under 2 years of age and 43% in children between two and five years old (Table 9). Influenza A was the most common finding among the elderly over 81 years old, with 87% of the findings. Only 3% of the findings were RSV in the elderly. The second most common finding among children between two and five years old was influenza A, 30%, and, among children under one year old, parainfluenza 1-3, 10%.

Adenovirus was found among both children and adults, but not in patients over 60 years of age. The few findings of enterovirus were mostly from specimens from small children. Measles virus was found during a small epidemic in 1999–2000 among adults between 21 and 40 years old. Herpes simplex was found in all age groups.

Annual variations were seen for influenza A and B, RSV, and parainfluenza 3.

Antigen detection with IF was only performed for antigens requested by the clinician on the referral form. For 8% of the culture findings, the corresponding IF was not requested by the clinician, and the finding was thus considered to be unexpected. Parainfluenza virus was the most common virus found in a sample but not requested by the clinician.

This retrospective analysis also allowed a methodological evaluation since both antigen detection and virus isolation were performed on the majority of samples, i.e. 79%. The IF sensitivity was 89% and 66% for influenza A and B, respectively, 74% for parainfluenza, and 29% for adenovirus. For virus isolation, the sensitivity was 96% for influenza A, 95% for influenza B, 75% for RSV, 91% for PIV, and 98% for adenovirus.
Table 9. Findings of respiratory virus in different age groups (paper I). All percentages are in comparison with all positive findings in the age group. Measles, herpes simplex virus, CMV and enterovirus are not shown.

<table>
<thead>
<tr>
<th>Age Group</th>
<th>Influenza A Virus</th>
<th>Influenza B Virus</th>
<th>RSV</th>
<th>PIV</th>
<th>Adenovirus</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–1 years (n=1507)</td>
<td>6%</td>
<td>1%</td>
<td>79%</td>
<td>10%</td>
<td>2%</td>
</tr>
<tr>
<td>2–5 years (n=246)</td>
<td>30%</td>
<td>7%</td>
<td>43%</td>
<td>8%</td>
<td>9%</td>
</tr>
<tr>
<td>6–20 years (n=157)</td>
<td>41%</td>
<td>18%</td>
<td>8%</td>
<td>8%</td>
<td>13%</td>
</tr>
<tr>
<td>21–60 years (n=569)</td>
<td>63%</td>
<td>14%</td>
<td>2%</td>
<td>3%</td>
<td>8%</td>
</tr>
<tr>
<td>61–80 years (n=431)</td>
<td>82%</td>
<td>10%</td>
<td>3%</td>
<td>2%</td>
<td>0%</td>
</tr>
<tr>
<td>81+ years (n=278)</td>
<td>87%</td>
<td>8%</td>
<td>3%</td>
<td>1%</td>
<td>0%</td>
</tr>
</tbody>
</table>

n = number of positive findings.
During the season 2002-2003 RSV was detected in 244 samples in the laboratory. 234 of them, representing 216 patients, could be amplified and sequenced and further analyzed by genotyping. Males were predominant, 61%, and most samples were from children under one year of age, 79%. Only six samples, 3%, were from patients over 50 years of age.

Strains belonging to both subgroups A and B were circulating during this season. Subgroup B was most frequent and was found in 152 of 234 strains (65%). The subgroup B strains could further be subdivided into two genotypes, GB3 and SAB1, with 137 (59%) and 15 (6%) strains, respectively. The 82 (35%) subgroup A strains could also be further subdivided into two genotypes, GA2 and GA5, with 25 (11%) and 57 (24%) strains, respectively.

RSV was detected from November to June during the studied season. The most common genotype, GB3, was found during the whole period. GA2 was found during January to March, GA5 from November to April, and SAB1 from January to April.

Two, three, or four consecutive sequential samples were collected from 12 patients. In eight of these patients, strains were obtained from both isolates and aspirates. No genetic variation was noted in the sequential sampling from the same patient, nor was any variations noted on comparing strains that were isolated and strains that were obtained by PCR from the same patient.

The strains from Stockholm were compared with other available strains from Great Britain, Kenya, Spain, Singapore, the USA and Uruguay, as well as with reference strains. The Swedish GA2 strains were closely related to strains from Kenya, Great Britain, and Singapore; the GA5 strains to strains from Kenya and Singapore, the GB3 strains to strains from Kenya and Great Britain, and the SAB1 strains to strains from Kenya.
5.3 PAPER III

A sensitive real-time PCR diagnostic platform for daily rapid detection of 15 respiratory viruses was developed, evaluated and put into operational use. The platform has a rational workflow and a short turnaround time.

The assays were evaluated using 585 stored pediatric NPA samples. In the pilot study realtime-PCR assays for adenovirus, bocavirus, HCoV-OC43, HCoV-229E, HCoV-HKU1, HCoV-NL63, HMPV, influenza A and B, picornavirus, and RSV A and B (11 viruses) were included. A previous analysis by IF and virus isolation had found viruses in 37% of the samples. With the new diagnostic panel, 57% of the samples were positive. The higher positive yield was due to more sensitive assays as well as to the findings of viruses that up to then had not been targeted in the routine diagnostic assays.

After the evaluation the diagnostic panel was modified by changing the picornavirus assay from SYBR green to probe detection and by developing real-time PCR assays for parainfluenza virus and enterovirus. The analyses of the 15 respiratory viruses were performed in 12 reactions. Three reactions were duplex assays: PIV type 1 with PIV type 3, RSV A with B, and PIV 2 with HCoV-229E.

A basic package consisting of RSV and influenza A and a complete package with all 15 viruses were offered to the clinician. We set the latest time for reporting results at 16.00 hours if the samples had arrived before 12.00. Samples arriving later than noon in urgent need for analysis of influenza A or B or RSV were analyzed using a rapid antigen test. If negative, the analysis was complemented with PCR the following day.

During the first 6 months in operational use, 1322 samples were analyzed, 791 (70%) with the basic package and 338 (30%) with the complete package. The majority of samples were nasopharyngeal aspirates (1129) followed by BALs and bronchial/tracheal secretions (193). In total, 618 viral findings were made (Table 10). The total frequency of viral findings with the basic package was 40%. Of all NPAs analyzed in the complete package, 57% were positive. The complete package was always used on respiratory samples from the lower respiratory tract, and 24% of these samples were positive.
Table 10. Findings of Respiratory Viruses During the First 6 Months of Clinical Use of the Diagnostic Platform

<table>
<thead>
<tr>
<th>Virus</th>
<th>Number of positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus</td>
<td>25</td>
</tr>
<tr>
<td>Bocavirus</td>
<td>20</td>
</tr>
<tr>
<td>Coronavirus</td>
<td></td>
</tr>
<tr>
<td>HCoV-HKU1</td>
<td>17</td>
</tr>
<tr>
<td>HCoV-229E</td>
<td>6</td>
</tr>
<tr>
<td>HCoV-OC43</td>
<td>3</td>
</tr>
<tr>
<td>HCoV-NL63</td>
<td>3</td>
</tr>
<tr>
<td>Enterovirus</td>
<td>21</td>
</tr>
<tr>
<td>Human metapneumovirus</td>
<td>3</td>
</tr>
<tr>
<td>Influenza A virus</td>
<td>65</td>
</tr>
<tr>
<td>Influenza B virus</td>
<td>132</td>
</tr>
<tr>
<td>Parainfluenza virus</td>
<td></td>
</tr>
<tr>
<td>PIV 1</td>
<td>7</td>
</tr>
<tr>
<td>PIV 2</td>
<td>1</td>
</tr>
<tr>
<td>PIV 3</td>
<td>1</td>
</tr>
<tr>
<td>Rhinovirus</td>
<td>71</td>
</tr>
<tr>
<td>Respiratory syncytial virus</td>
<td>243</td>
</tr>
</tbody>
</table>
Clinical data from patients with single findings (92 patients with 94 episodes of respiratory tract infection) were compared to data from patients with multiple findings (37 patients and episodes). Only multiple infections involving RSV, coronavirus, or rhinovirus were included. All children single-infected with coronavirus or rhinovirus were included as controls and, for RSV, age-matched single-infected children. Demographic data were obtained for the entire study population of 585 children.

Patients with multiple findings were older (mean 12.7 months) than patients with single findings (mean 8.8 months). Including all RSV single-infected children from the evaluation material, the mean age was 5.7 months (P < 0.01).

There were no statistically significant differences in the duration of hospitalization, i.e. 3.4 days for those with a multiple finding and 3.3 for those with a single finding. Children with coronavirus and one more virus were hospitalized 3.4 days compared to 2.9 days for children infected only with coronavirus, but this was not statistically significant either.

CRP levels were more likely to be above 70 mg/L in multiple-infected children and children with multiple infections were also more likely to have a chest x-ray examination than children with single infections. These differences were not statistically significant.

No differences were noted between the two groups in oxygen treatment or admittance to the intensive care unit.

On studying the subgroup of children with comorbidities and comparing those with multiple findings with those with single findings, no significant differences in the above-mentioned parameters were noted.

The most common diagnosis at discharge in the whole series was wheezing, except among children infected only with coronavirus, in whom URTI was most common. Croup was diagnosed in three patients: interestingly, two of these patients were single-infected with HCOV-NL63, a virus associated with croup in some studies [Rihkanen et al., 2008; van der Hoek et al., 2005].
6 DISCUSSION

6.1 ASPECTS OF THE EPIDEMIOLOGY OF VIRAL RESPIRATORY TRACT INFECTIONS

6.1.1 General aspects
The new molecular diagnostic methods, the newly identified viruses, the SARS epidemic, and the emerging avian influenza have all contributed to an increased interest in viral respiratory infections. Our knowledge of the epidemiology of respiratory infections has been expanded by several prospective and retrospective studies. The frequencies of viral findings differ between these studies, depending on the inclusion criteria, the year and number of years studied, the specificity and sensitivity of the diagnostic methods used, and the age of the population.

In paper I the diversity of respiratory virus infections during six seasons was studied in hospitalized patients using antigen detection and virus isolation. The positive rate for children under five years of age was 42%. In 58% of the samples no virus was found. Explanations of this might be the broad inclusion criteria, a bacterial infection instead of a virus infection, or a suboptimal sampling technique. Another explanation is of course that many viruses were not targeted by virus isolation and IF. The results and conclusions from paper I formed the background for the work in paper III.

In paper III a rapid real-time PCR platform for 15 viruses was evaluated using frozen pediatric specimens and set into operational use. The rate of positive findings increased from 37% using virus isolation and antigen detection to 57% using the PCR platform. This increase in positive yield was mostly due to detection of those viruses that had not been targeted using our earlier methods.

The work in papers I and III was based on hospitalized patients and showed that a broad diagnostic method was necessary to get the best possible diagnostic yield for patients with respiratory infections admitted to hospital. In order to detect the true frequency of respiratory tract infections in the population, prospective studies including all ages and covering more than one year have to be done. Such studies were conducted from the 1940s to the 1960s especially in North America and contribute to our knowledge of respiratory viral infections in the community [Monto, 1994]. But since, for obvious reasons, the newly identified viruses were not included in these population-based studies, they have to be repeated.

Some prospective population-based studies have been presented in the more recent literature. Lambert and coworkers conducted a 12-month prospective study on the community epidemiology of 10 respiratory viruses in a cohort of preschool-aged children in Melbourne. They found that picornaviruses were the most common virus finding, that HMPV and HCoV-NL63 were important pathogens particularly among children in day care, and that influenza virus and adenovirus caused the most severe illnesses [Lambert et al., 2007]. In a study by Regamey and coworkers in Switzerland, 197 infants were followed during their first year of
life. An etiologic agent was found in more than 80% of the cases of respiratory illness. Rhinovirus, bocavirus, coronavirus, and HMPV were found in more than half of the cases. RSV was found in only 15% of the samples but was, on the other hand, associated with the most severe illnesses [Regamey et al., 2008]. These population-based studies support our finding that it is important to include the newly detected viruses in the diagnostic methods for daily use.

**6.1.2 RSV in the elderly**

One of the aims of paper I was to study the impact of RSV among hospitalized elderly persons. Only 3% of findings in patients over 60 years of age were RSV in this series.

Several studies have found that RSV is an important respiratory virus among the elderly [Falsey et al., 2005; Falsey and Walsh, 2000; Fleming and Cross, 1993]. Jansen and coworkers analyzed the statistics on respiratory disease and excess death rates and found that morbidity and mortality in persons over 65 years of age peaked when RSV activity was highest in the community. This peak could not be explained by a peak in influenza activity [Jansen et al., 2007].

The findings in our retrospective analysis (paper I) do not support the view that RSV is a common finding among hospitalized elderly persons, as reported elsewhere. The study population is different from other studies considering this specific problem. Many studies have prospectively included elderly persons living in long-term care facilities [Falsey and Walsh, 2000]. Other studies have estimated mortality and morbidity indirectly using the reported number of cases in a surveillance system [Jansen et al., 2007; Thompson et al., 2003].

In the retrospective analysis in paper I, virus isolation was performed on nearly all samples. Immunofluorescence, which is a more sensitive method for detection of RSV than virus isolation, was performed only if requested. There are reports that RSV is best detected by PCR and not by antigen detection with IF in the elderly [Falsey et al., 2002a; Falsey and Walsh, 2006]. This could be due to a lower titer of virus in the nasopharynx in the elderly than in children [Falsey and Walsh, 2000] The low frequency of RSV in the elderly in the series in paper I could be due to a lower sensitivity of IF and virus isolation in this age group.

During one season (2000-2001) IF for RSV was performed on all samples in an attempt to increase the findings of RSV in adults and the elderly (Table 11). During this season more RSV infections were diagnosed by IF than during any of the five seasons before (unpublished data). It is notable that the RSV epidemic in 2000–2001 was a minor one. These results are in agreement with published data indicating that the diagnosis of RSV among the adults and elderly may be underestimated.
Table 11. Findings of RSV in Relation to the Use of IF for Diagnosis.

<table>
<thead>
<tr>
<th>Year</th>
<th>RSV IF/No. of NPAs (%)</th>
<th>RSV findings/No of NPAs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1995-2000</td>
<td>108/1214 (9%)</td>
<td>8/1214 (0.7%)</td>
</tr>
<tr>
<td>2000-2001</td>
<td>202/223 (90%)</td>
<td>8/223 (4%)</td>
</tr>
</tbody>
</table>

The need for an RSV vaccine not only for children but also for the elderly has been discussed. Even though there are convincing data from particularly North America and Great Britain that RSV has a great impact also among the elderly, this has to be confirmed by prospective studies in other parts of the world.

6.1.3 RSV epidemics

Even though a biennial pattern of RSV epidemics has been reported in several published studies, this has been the subject of further discussion [Eriksson et al., 2002; Mlinaric-Galinovic et al., 2008; Waris, 1991]. It is difficult to see this distinct biennial pattern in data provided by the Swedish Institute for Disease Control (Figure 1). This is, however, more clearly seen when studying data from the geographic area of northern Stockholm (Figure 2) (Paper I). It seems likely that the biennial pattern is more distinct in data from a smaller geographic area than in data from laboratory reports from a whole country such as Sweden.
Figure 1. Laboratory findings of RSV in Sweden as reported by the Swedish Institute for Disease Control

![Graph showing laboratory findings of RSV in Sweden]

Vecka = Week, Antal = Number of cases

Figure 2. Alternating early major epidemics and later minor epidemics in Stockholm 1992–2000. Major epidemics (in red) (starting early in autumn, uneven years), minor epidemics in blue (starting later in autumn, even years). Based on weekly reports of RSV findings from the laboratory.

![Graph showing alternating major and minor epidemics in Stockholm]
6.1.4 Influenza in the youngest
RSV is the most common LRTI in small children. However, the state of knowledge on the impact of influenza in children has attracted more attention during recent years [Esposito et al., 2008; Iskander et al., 2007]. The finding that influenza was the second most common virus among children 2-5 years of age in paper I and the third most common virus in paper III is in agreement with this phenomenon.

In the United States influenza vaccination is recommended not only to the elderly and to defined risk groups, but also to children between 6 months and 18 years of age [Fiore et al., 2008]. Acute otitis media is a common complication after influenza in childhood, and one advantage of a general influenza vaccination is the possible decrease in otitis media [Jansen et al., 2008]. Another reason for vaccination of children is to limit the spread of influenza from children to adults.

6.1.5 Bocavirus
The importance of bocavirus as a respiratory pathogen has been established since the virus was first described in 2005 [Allander et al., 2007; Jartti et al., 2007]. So far, wheezing is the clinical illness that is most closely associated with bocavirus. The virus has often been found as one of two or three pathogens in a respiratory specimen, leading to difficulties in interpretation of the results [Schildgen et al., 2008]. The use of quantitative or semi-quantitative detection methods appears to be useful for addressing this issue. Bocavirus has been detected at a higher viral load when found as a single pathogen than when found in a multiple infection [Allander, 2008]. This was also found in paper III (Table 12).

Case reports of symptomatic primary infection with bocavirus have been published and the concept that a high bocavirus load is associated with primary infection has been confirmed by serology [Kantola et al., 2008]. We have described a case of a 4-year-old patient with a history of infection associated with wheezing, who had a severe ECMO-treated respiratory tract infection. Bocavirus was found at high copy numbers in the respiratory tract and also in the blood, and the patient had IgM antibodies against bocavirus [Allander et al., 2008].

Considering the increasing evidence that bocavirus might be an important respiratory pathogen, it seems both reasonable and important to include bocavirus in a respiratory diagnostic panel.
Table 12. Ct values for bocavirus in single and multiple findings (paper III). Comparison of the Ct values between the two groups, P = 0.15 (Mann-Whitney). On excluding the outlier (Ct 37.06) among the single findings; P < 0.01. This child had been admitted a few days earlier for a rhinovirus infection.

<table>
<thead>
<tr>
<th>Multiple findings (mean 32.4)</th>
<th>Single finding (mean 25.7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>31.88</td>
<td>23.91</td>
</tr>
<tr>
<td>34.4</td>
<td>20.51</td>
</tr>
<tr>
<td>29.62</td>
<td>19.89</td>
</tr>
<tr>
<td>31.77</td>
<td>32.85</td>
</tr>
<tr>
<td>36.54</td>
<td>14.22</td>
</tr>
<tr>
<td>34.51</td>
<td>34.56</td>
</tr>
<tr>
<td>32.32</td>
<td>22.79</td>
</tr>
<tr>
<td>30.51</td>
<td>37.06</td>
</tr>
<tr>
<td>24.5</td>
<td></td>
</tr>
<tr>
<td>35.97</td>
<td></td>
</tr>
<tr>
<td>32.73</td>
<td></td>
</tr>
<tr>
<td>34.15</td>
<td></td>
</tr>
</tbody>
</table>

6.1.6 Rhinovirus and coronavirus

The three most frequent common cold viruses, rhinovirus, HCoV-OC43, and HCoV-229E, have not been frequently diagnosed before now in the clinical viral laboratory setting. Both coronavirus and rhinovirus were studied in some of the earlier prospective population studies and found to be important causes of the common cold [Monto, 1994].

During recent years, however, rhinovirus has been reported to be a more important pathogen than earlier believed. Rhinovirus has also been found to be an important pathogen in exacerbations of chronic bronchitis and cystic fibrosis and in wheezing in all age groups [Johnston et al., 1995; Nicholson et al., 1993]. The importance of rhinovirus in infants is also under debate, and rhinovirus may be underestimated as a cause of a severe respiratory infection in small children [Brownlee and Turner, 2008; Mackay, 2008; Miller et al., 2007].

The findings of coronaviruses (17 HCoV-NL63, 17 HCoV-OC43, one HCoV-HKU1 and no HCOV-229E) in paper III is in agreement with other findings of coronaviruses. In paper IV the coronavirus infection was predominantly associated with URTI. Wheezing was much less frequently observed compared to in the rhinovirus and RSV subgroups. In most studies HCoV-HKU1 has only been found in a few percent, and it is also this virus that has the weakest association with a defined respiratory illness [Woo et al., 2005]. HCoV-NL63 is
clearly associated with croup, and this was also found in paper IV [Rihkanen et al., 2008; van der Hoek et al., 2005].

There are many studies confirming the importance of rhinovirus and the two previously known coronaviruses in both prospective population-based studies and among hospitalized patients [Falsey et al., 2002b; Kusel et al., 2006; Kuypers et al., 2007; Lambert et al., 2007; Lau et al., 2006; Miller et al., 2007; Regamey et al., 2008]. Even if most patients with rhinovirus and coronaviruses have a mild self-limiting upper respiratory tract infection, there are patients diagnosed with more severe respiratory tract infections with these viruses and no other microbiological pathogen. It is reasonable to believe that many of these patients suffer from symptoms caused by these pathogens.
6.2 ASPECTS OF DIAGNOSTIC METHODS FOR VIRAL RESPIRATORY INFECTIONS

In the first paper of this thesis the conventional methods for diagnosing respiratory viruses, virus isolation and antigen detection with IF were used. In the third paper a molecular diagnostic platform was developed and implemented. The old methods were fully replaced by the new platform.

6.2.1 Sensitivity
For RSV there was a slight rise in sensitivity using PCR, but the rise was more pronounced for adenovirus and influenza B virus compared to virus isolation and IF. The presence of influenza B virus in a respiratory specimen certainly indicates that the patient has a symptomatic influenza infection. Adenovirus is known to be secreted in stool for a long period of time after infection, and the finding of adenovirus in a respiratory sample has hitherto been associated with illness in the patients [Monto, 1994]. It has to be proven if the finding of adenovirus in a respiratory sample using molecular methods has the same impact as the finding with the old methods [Jartti et al., 2008a].

6.2.2 Decreased processing time
Sensitive and rapid testing for RSV and influenza with IF has been available for many years. When handling a limited number of specimens a result might be reported in about 1-2 hours. The sensitivity of IF is good enough for influenza virus and RSV, but a lot poorer for adenovirus and parainfluenza virus. With daily molecular testing, a result for all respiratory viruses could be reported the same day or the day after, compared to after one or two weeks with virus isolation.

The more rapid finding of adenovirus by molecular methods could prevent unnecessary antibiotic treatment. The importance of knowing the causative agent is difficult to measure in money savings, but is of importance both for the clinician and the patient.

6.2.3 Quantification
Quantifications of CMV, EBV, and adenovirus in blood are established methods, mostly used in immunocompromised patients. The use of preemptive therapy is one of the reasons for monitoring patients for CMV [Meijer et al., 2003; Strippoli et al., 2006]. The use of real-time PCR with probe detection makes it possible to quantify respiratory viruses as well.

Levels above 500,000 copies/mL of CMV in BAL have been shown to be associated with respiratory illness in the lung-transplanted patient [Drew, 2007]. However, CMV is a latent virus and low levels of it in respiratory samples will not indicate symptomatic infection. Respiratory viruses are, with the possible exception of bocavirus, not known to persist in the human body. Therefore, quantification may not be as necessary for these viruses as for latent or persisting viruses, and qualitative tests would be sufficient.
Quantification may be useful for interpreting the clinical significance of multiple findings. Bocavirus, as described above, is known to be present in higher viral loads when found as a single pathogen. For other respiratory viruses, the state of knowledge is incomplete concerning levels of viral load in multiple findings.

Quantification may also have an impact in sequential sampling from the same patient. During a period of illness it is reasonable to believe that the viral load will decrease. For immunosuppressed patients, it could be of importance to know if the patient’s immune system can eliminate the virus.

The NPA itself is not of a constant volume. After suction, the tube is rinsed with a small amount of NaCl, and the Ct level may vary with the volume of NaCl. However, the amount of NaCl will not vary by more than 2-3 mL, and this is less than a tenfold variation in volume. Therefore, changes of the order of one or two logs (3-6 steps in Ct value) could indicate true increases or decreases in the viral load.

### 6.2.4 Sequencing and genotyping

Sequencing and genotyping can be applied to most respiratory viruses in order to better understand the epidemiologic pattern and the impact of different genotypes on the severity of illness. RSV strains from different years and several countries have recently been genotyped. The same RSV genotype could be found in different parts of the world with many years in between. Both subgroups, A and B, could be present during the same epidemic and, in 2002–2003 in Stockholm, at least four genotypes were circulating. One of these genotypes has been circulating for 20 years in Sweden [Rafiefard et al., 2004]. The different genotypes do not appear to have the same impact on the magnitude of the RSV epidemic as the different influenza strains. Factors such as local immunity and transmission of maternal antibodies are believed to be of greater importance.

The sensitivity of the PCR assays is dependent on the primers and probes binding to the complementary DNA (cDNA). Even small changes in one or two bases can influence the assay and give a false negative result. This problem is one of the disadvantages of PCR. Thorough surveillance of respiratory virus strains by sequencing is required in order to find minor and major changes in the viral genome. The laboratory and the clinician also have to cooperate closely, so that a cluster of respiratory tract infections without a diagnosis can be further analyzed by alternative PCR assays and other diagnostic methods.
6.3 ASPECTS OF THE INTERPRETATION OF RESULTS

6.3.1 Secretion after and before infection

The increased sensitivity when using molecular diagnostic methods makes it more likely that a virus will be found for a longer period of time after infection.

Most studies on the shedding of respiratory viruses have been done using conventional methods such as virus isolation and IF. By these methods, most respiratory viruses are found to be present in an NPA for 1-2 weeks after the onset of symptoms. The time during which a respiratory virus could be detected may be longer if using PCR assays. This assumption is important when interpreting results. There is also evidence that some viruses can be present in respiratory specimens before the onset of symptoms [Jartti et al., 2004; Winther et al., 2006]. If the time during which shed virus is detectable is prolonged by using molecular assays, there will be opportunities for multiple findings in respiratory specimens. In such cases, it is likely that one of the viruses is the causative agent and that the other one remains from an earlier infection.

6.3.2 Viral findings in asymptomatic patients

Influenza viruses, PIV and RSV are not frequently, or even almost never, found in asymptomatic patients [Chanock et al., 2001; Collins et al., 2001; Wright and Webster, 2001]. Bocavirus appears to be present at low viral loads in multiple infections quite frequently and is believed to be shed for a long time in some individuals [Schildgen et al., 2008]. Rhinovirus has also been reported to be present in asymptomatic patients [Jartti et al., 2008a; Nokso-Koivisto et al., 2002; Winther et al., 2006].

An accepted definition of the the normal microbial flora in bacteriology implies that bacteria are present but not pathogenic. Is there a normal microbial flora of viruses in the respiratory tract? Or rather, is there a normal microbial flora of viruses in small children [Jartti et al., 2008a]?

One of the differences in comparison to the bacteriological normal microbial flora is that all viruses that could be present in an asymptomatic patient are mostly pathogens. Therefore, it is possible that, for instance, a finding of rhinovirus in a seriously ill infant could be the finding of the pathogen, but it could also be a harmless bystander.

The interpretation of the sole or multiple finding of coronavirus, rhinovirus, and bocavirus is still a challenge to both the clinical virologist and the clinician.

6.3.3 Multiple findings

In the first paper in this thesis less than 1% of all infections were multiple findings. In the third paper one or more viruses were found in 7% of all samples. This increase in multiple findings is not only due to the increased sensitivity, but also to the increased possibility of detecting viruses that have not been targeted with the previous diagnostic methods.
There are many possible interpretations of a multiple finding. Two or more viruses could be present in a respiratory specimen in a true multiple infection, with all viruses causing symptoms. There could also be overlapping infections; the patients could have recovered from an RSV infection but shortly thereafter picked up a rhinovirus infection [Jartti et al., 2008b]. A sensitive PCR assay would detect both viruses. A third possibility is prolonged shedding in, for instance, immunocompromised patients. So far, the state of knowledge of secretion time is limited when it comes to using molecular diagnostic methods.

There is evidence for the possibility that multiple infections result in more severe symptoms than single infections [Foulongne et al., 2006; Konig et al., 2004; Richard et al., 2008; Semple et al., 2005], but there are also studies showing no difference in clinical outcome [Canducci et al., 2008; Wolf et al., 2006]. In paper IV, children with multiple findings were significantly older than those with single findings. An explanation for the older age in children with multiple findings might be a greater exposure to circulating viruses with increasing age. No statistically significant differences could be identified regarding hospitalization time, the outcome of chest x-rays, or levels of CRP, but there were trends toward higher CRP levels and more frequent use of chest x-rays in patients with a multiple finding. Multiple findings in the studied group of children may, in fact, reflect sequential infections rather than true simultaneous infections.
7 CONCLUSIONS

The frequency of “old” viruses such as adenovirus, influenza virus, parainfluenza virus and RSV was quite similar in children studied with conventional methods such as virus isolation and antigen detection in paper I and with PCR in paper III. In paper III the “new” viruses (HCoV-NL63, HCoV-HKU1, bocavirus, and HMPV) contributed to the increased diagnostic yield as well as the viruses that have been known hitherto but not targeted by the conventional methods (HCoV-OC43, HCoV-229E, and rhinovirus). Molecular diagnostic methods for respiratory viruses are feasible for the diagnosis of respiratory virus infections and should be used preferentially in hospitals treating severely ill patients in order to get as high a diagnostic yield as possible.

The clinical symptoms of the “old” viruses are well-known. In order to better interpret the findings of coronavirus, rhinovirus, HMPV, and bocavirus in patients with severe respiratory disease, more prospective studies, if possible, with control groups, are necessary.

Children with multiple findings were significantly older than those with a single finding. There was no significant difference between the groups regarding hospitalization time, diagnosis, CRP levels, x-ray findings, or oxygen therapy. This indicates that multiple findings in hospitalized children are sooner findings of sequential infections than true multiple infections. The clinical outcome of “true” multiple infections needs to be further evaluated by prospective studies with frequent sampling.

The low number of RSV findings among the hospitalized elderly in paper I indicates either that RSV is not common among elderly persons in Sweden or that RSV infection appears before admission to the hospital, or that the RSV-infected elderly are not necessarily admitted to hospital. Another explanation is that more sensitive methods than IF and virus isolation have to be used in the elderly. Since vaccines against RSV in the elderly are being discussed and are under development, the impact of RSV in, for instance, long-term facilities needs to be more thoroughly studied in more geographic areas.

The similarity between RSV strains from Sweden and other parts of the world supports the current theories for RSV epidemiology suggesting that local factors, such as local immunity in the population, are more important for the outcome of RSV-epidemics than globally circulating strains.
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9 REFERENCES


