SEVERE VIRAL RESPIRATORY TRACT INFECTIONS IN CHILDREN

Samuel Arthur Rhedin

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Nomina si nescis, perit et cognito rerum
– Carl Linnaeus
ABSTRACT

Respiratory tract infections (RTIs) are estimated to cause 703,000 deaths annually in children below five years. The majority of RTIs in children are caused by viruses, yet the number of antivirals approved for treatment of these infections is very limited. Moreover, it is sometimes complicated to distinguish between bacterial and viral RTIs, which results in overuse of antibiotics. The aim of this thesis is to improve the understanding of the causative role of respiratory viruses in children with severe RTI, with the long-term goal to improve diagnostics, facilitate the development of new antiviral drugs and reduce unnecessary antibiotic use. To achieve this, a number of specific objectives have been assessed.

The spread of the Influenza A H1N1 (pdm09) i.e. the swine flu pandemic was slower than expected when it reached Europe during Spring 2009. This was suggested to be due to negative viral interference by circulating rhinovirus (RV). In Paper I, children with influenza-like illness were assessed during the swine flu pandemic in 2009. Co-infections were specifically assessed in influenza-positive patients with regard to disease severity. No significant difference was found between patients with single versus viral co-infection. Co-infection with influenza and RV was not uncommon, which contradicted the proposed hypothesis of viral interference. Moreover, the study showed that several different viruses were present in the children with suspected influenza, underscoring the overlap of disease presentation of different respiratory viruses.

PCR is a very sensitive method for detecting viruses, yet the significance of a finding in upper respiratory specimens has been questioned. In Paper II, we assessed the role of viruses in acute respiratory illness in a case-control study. Respiratory syncytial virus (RSV), human metapneumovirus (hMPV) and parainfluenza virus were highly associated with acute respiratory illness. In contrast, detection of other viruses was common in asymptomatic controls, showing the complexity in interpreting PCR-positivity for these viruses.

Community-acquired pneumonia (CAP) is a disease that traditionally has been considered a predominantly bacterial disease. Nevertheless, successful immunization against the two major bacterial causes, Streptococcus pneumoniae and Haemophilus influenza, has contributed to a declining incidence of the disease and has likely also led to a relative increase of other etiologic agents. In Paper III, the role of viruses in CAP was assessed in another case-control study. Viruses were detected in the majority of cases and RSV, hMPV and influenza were highly associated with CAP. The study suggests that viruses have a major role in childhood CAP and indicates that viral CAP is an underdiagnosed disease.

Viral RTIs affect also immunosuppressed children. Neutropenia is a common adverse effect in children receiving chemotherapeutic treatment for malignancies. The condition highly increases the risk for septicemia, and fever is sometimes the only symptom. However, in the majority of episodes of febrile neutropenia, no causative agent can be identified. In Paper IV, respiratory viruses were assessed in immunosuppressed children during episodes of febrile neutropenia. Interestingly, respiratory viruses were detected in almost half of the episodes, whereas laboratory confirmed septicemia was infrequent (9%). Moreover, the majority of children had cleared their virus at follow-up suggesting a causal relationship between the detected viruses and the episodes of febrile neutropenia.

This thesis has contributed to an improved understanding of the role of viruses in severe RTIs in children stressing the urgent need for new diagnostic tests that better distinguish between viral and bacterial disease. It also forwards the need for improved treatment options and new vaccines against viral RTIs in children.
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LIST OF ABBREVIATIONS

ARI  acute respiratory illness
CAP  community-acquired pneumonia
CI   confidence interval
CRP  C-reactive protein
CT   cycle-threshold
DNA  deoxyribonucleic acid
ER   emergency room
EV   enterovirus
Flu  influenza
FN   febrile neutropenia
GAS  group A streptococci
H1N1 influenza A H1N1(pdm09)
HAdV human adenovirus
HBoV human bocavirus
HCoV human coronavirus
Hib  *Haemophilus influenzae* type B
hMPV human metapneumovirus
Ig   immunoglobulin
ILI  influenza-like illness
IMCI International Management of Childhood Illnesses
LRTI lower respiratory tract infection
MERS Middle East respiratory syndrome
MRSA methicillin-resistant *Staphylococcus aureus*
MxA myxovirus resistance protein A
NGS next-generation sequencing
NPA nasopharyngeal aspirate
OR  odds ratio
PCR real-time polymerase chain reaction
PCV pneumococcal conjugate vaccines
PIV parainfluenza virus
RNA ribonucleic acid
RPA recombinase polymerase amplification
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSV</td>
<td>respiratory syncytial virus</td>
</tr>
<tr>
<td>RTI</td>
<td>respiratory tract infection</td>
</tr>
<tr>
<td>RV</td>
<td>rhinovirus</td>
</tr>
<tr>
<td>SARS</td>
<td>severe acute respiratory syndrome</td>
</tr>
<tr>
<td>SES</td>
<td>socio-economic status</td>
</tr>
<tr>
<td>TRAIL</td>
<td>tumor necrosis factor-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>URTI</td>
<td>upper respiratory tract infection</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
</table>
1 BACKGROUND

1.1 RESPIRATORY TRACT INFECTIONS

Respiratory tract infection (RTI) is, second to neonatal complications, the most common cause of death in children and is estimated to cause 703,000 deaths annually in children below five years (1). Mortality is skewed to low- and middle-income countries yet RTIs attribute to significant morbidity also in high-income countries (1). Several pathogens including viruses, bacteria, and fungi are capable of infecting the respiratory tract yet the focus of this thesis is on viral RTIs. Advances in diagnostic methods such as the development of molecular-based clinical tests have revolutionized viral diagnostics and recently several novel viruses have been discovered (2–5). There is a lack of understanding of the epidemiology, the clinical presentation and significance of different respiratory virus infections, particularly of those that were only recently discovered (2).

1.1.1 Anatomy of the Respiratory Tract

The respiratory tract is commonly divided into the upper and lower respiratory tract. Most of the respiratory tract is covered by ciliated pseudostratified columnar epithelial cells whereas the oral cavity, the tonsils and epiglottis have a stratified squamous non-keratinized epithelium, possibly to withstand the abrasion associated with ingestion of food (figure 1). Conformational differences in the glycans covering the epithelial cells partly explain the tissue tropism and species-specificity for different pathogens (6).

![Figure 1. Epithelium in the respiratory tract.](image)

A. Ciliated pseudostratified columnar or respiratory epithelium in the lower respiratory tract.
B. Stratified squamous epithelium in the oral cavity.

1.1.1.1 Upper Respiratory Tract

The upper respiratory tract consists of the oral and nasal cavities, the sinuses, the pharynx (oropharynx, nasopharynx, and laryngopharynx) and the larynx (7). The ear cavities are sometimes included given their anatomical connection to the nasopharynx through the Eustachian tube. The lymphoid tissue of Waldeyer's tonsillar ring (including the pharyngeal tonsil or “adenoid” in the nasopharynx, the lingual tonsil on the posterior tongue, two palatine tonsils in the oropharynx and two tubal tonsils at the opening of the Eustachian tube) and the epiglottis are other important structures in the upper respiratory tract. Bacterial colonization of
the upper respiratory tract is common in children, for instance approximately 30% of Swedish children and 60–90% of children in low-income countries are carriers of *Streptococcus pneumoniae* (8,9). Other important colonizing bacteria in children are *Haemophilus influenzae*, *Staphylococcus aureus*, group A *Streptococci* and *Moraxella catharralis* (8). Significant differences in the microbiota are seen between breastfeeding children and children receiving formula (10).

The existence of viral colonization is debated. Plenty of respiratory viruses are detected in the respiratory tract of asymptomatic children and longitudinal studies have reported persistence of certain viruses for several weeks (11–13). Nonetheless, as viruses are intracellular organisms and as such dependent on the cellular machinery to replicate and persist, some argue that asymptomatic detection should not be considered colonization but rather subclinical or latent infection (12).

1.1.1.2 Lower Respiratory Tract
The lower respiratory tract consists of the trachea, the bronchi, the bronchioli and the lungs (alveoli and lung parenchyma). The lower respiratory tract has historically been considered a sterile site but metagenomic studies of lower respiratory specimens have revealed a plethora of resident microorganisms, thus challenging this old dogma (14). A certain confirmation of sialic acid-linked glycans, the target molecule or receptor for influenza virus, is found in the alveolar cells. This conformation, alpha(2,3), contrasts to the alpha(2,6) confirmation that is widespread in the upper respiratory tract (6). The impact of this was evident during the swine flu pandemic in 2009 where a certain mutation of the influenza virus was overrepresented in patients with severe lower respiratory tract infections in Norway (15). It was later shown that this mutation increased the virus affinity for alpha(2,6), which could have contributed to the high pathogenicity (15).

1.1.1.3 Respiratory Specimens
A variety of respiratory specimens are obtained for diagnostic purposes depending on the severity of the disease and focus of the infection (table 1). The nasopharyngeal aspirate is considered gold-standard for upper respiratory infections but is also commonly used in diagnostics of lower respiratory infections under the assumption that the infecting pathogen entered the lower respiratory tract through micro-aspiration. Pleural fluid sampling, blood culture, bronchoalveolar lavage, lung aspirates and lung biopsies/autopsies are considered the gold standard for lower respiratory infections, but these specimens are either not feasible to obtain or lack sensitivity in children (16).
Table 1. Specimens for Diagnosing Respiratory Infections in Children

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Application</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nasal swab</td>
<td>Rapid tests (Respiratory syncytial virus, influenza)</td>
<td>Minimal discomfort for the child. Commonly used for rapid testing of respiratory syncytial virus and influenza.</td>
</tr>
<tr>
<td>Nasopharyngeal aspirate/swab</td>
<td>Upper respiratory tract infections&lt;br&gt;Lower respiratory tract infections</td>
<td>Gold standard sampling for influenza, bronchiolitis, atypical pneumonia and whooping cough.</td>
</tr>
<tr>
<td>Oropharyngeal/throat swab</td>
<td>Tonsillitis</td>
<td></td>
</tr>
<tr>
<td>Expectorated sputum</td>
<td>Pneumonia</td>
<td>Hard to obtain from young children. Contaminated by pathogens from the upper respiratory tract.</td>
</tr>
<tr>
<td>Induced sputum</td>
<td>Pneumonia</td>
<td>Acquired through inhalation of saline. Sensitive and tolerable for the child. Contaminated by pathogens from the upper respiratory tract.</td>
</tr>
<tr>
<td>Pleural fluid</td>
<td>Empyema/parapneumonic effusion</td>
<td>Diagnostic and therapeutic intervention in pneumonia with pleural effusion.</td>
</tr>
<tr>
<td>Bronchoalveolar lavage</td>
<td>Severe lower respiratory tract infections</td>
<td>Invasive, mainly used in the intensive care unit setting.</td>
</tr>
<tr>
<td>Lung aspirate</td>
<td>(Severe pneumonia)</td>
<td>Rarely used in the clinic due to the invasive nature. Advantage of direct sampling from the lungs. Used widely in etiological studies during the 60s - 80s. Performed routinely in some African countries with reportedly low complication rate (17).</td>
</tr>
<tr>
<td>Lung biopsy</td>
<td>Severe lower respiratory tract infections with treatment failure (18).</td>
<td>Only recommended in case of treatment failure in patients with severe pneumonia. Can be performed open or by thoracoscopy.</td>
</tr>
<tr>
<td>Autopsy</td>
<td>Fatal respiratory infections</td>
<td>Advantage of direct sampling from the infectious focus.</td>
</tr>
<tr>
<td>Urine antigen tests</td>
<td>(Pneumonia)&lt;br&gt;Available for pneumococci, legionella.</td>
<td>Not recommended for routine use in children due to high colonization rates of the nasopharynx, which yields false-positive results (18).</td>
</tr>
<tr>
<td>Blood culture</td>
<td>Severe pneumonia</td>
<td>Poor sensitivity in children.</td>
</tr>
<tr>
<td>Fecal tests</td>
<td>Research</td>
<td>Several respiratory viruses are detectable in fecal samples (19,20).</td>
</tr>
</tbody>
</table>
1.1.2 The Immune System

Immunity comes from the Latin word *immunis* meaning “exemption from military service or tax payment.” In medicine, it refers to the long-lasting protection against infection, which is maintained by specific immune cells in the body as well as by physical barriers. The immune system is commonly divided into the innate and the adaptive immune system.

1.1.2.1 The Innate Immune System
The innate immune system is the first line of defense. It consists of physical barriers, as well as a variety of cells specialized at sensing evolutionarily preserved patterns commonly found on microbes. The response is fast and constantly prevents us from being infected by microbes in the environment. Tightly arranged epithelial cells secrete antimicrobial peptides preventing microbes from penetrating the skin and the respiratory tract (21). Neutrophil granulocytes are specialized at phagocytizing, i.e. ingesting bacteria and dead tissue. Eosinophil granulocytes defend against parasites whereas the role of basophil granulocytes is less well understood. Both eosinophils and basophils are involved in the pathology of asthma and allergies. Monocytes such as macrophages and dendritic cells are specialized at presenting fragments of ingested pathogens to cells from the adaptive immune system thus providing a crucial link between the two parts of the immune systems. They also produce pro-inflammatory cytokines (IL-1, IL-6, TNF-α, etc) and chemokines. Natural killer cells are important in the defense against viruses and cancer cells. The complement system consists of plasma proteins that act as opsonins, i.e. they attach to intruding microbes to facilitate ingestion by phagocytizing cells. They also induce inflammation and kill microbes by forming pores in their membranes. Acute-phase proteins are peptides that rapidly increase in concentration in the blood following an infection and can opsonize certain pathogens. Some of these proteins such as C-reactive protein (CRP) are measured in the blood as markers of inflammation.

1.1.2.2 The Adaptive Immune System
The adaptive immune system consists of lymphocytes, highly specialized immune cells that create a tailor-made response to the intruding pathogen, once an infection is established. The response increases over time and usually provide long-lasting immunity against the pathogen. B-lymphocytes derive from the bone marrow and circulate in the blood as well as in the lymph nodes and in the spleen. They are important in the fight against mainly extracellular pathogens and can evolve into plasma cells, that produces immunoglobulins (Ig) or antibodies, soluble plasma proteins specifically targeting intruding pathogens. Antibodies from the mother are transfused to the child at birth (mainly IgG) and through breastfeeding (IgA) providing an efficient immune system to the newborn. Indeed, breastfeeding children acquire fewer infections as compared to those receiving formula (22). Most B-lymphocytes are short-lived, however, some evolve into memory cells after an infection that help maintain a long-lasting immunity to the pathogen. T-lymphocytes are specialized in fighting pathogens hiding inside human cells such as viruses and intracellular bacteria. This is maintained by induced cell death of the infected cells as well as by the production of cytokines stimulating cells in the innate immune system. All immune cells are initially produced in the yolk sack and liver during the embryonic phase but the production is relocated to the bone marrow after birth. Several immunological blood tests are routinely performed in the clinic, yet studies of the blood do not
always reflect what is happening in the peripheral tissue where most immune cells reside and where most of the host-pathogen interaction take place (23).

1.1.3 Upper Respiratory Tract Infections

Lack of immunity and high exposure at daycare centers and schools make upper respiratory tract infections (URTI) common in children. A longitudinal community surveillance study in Utah reported that children <5 years experienced respiratory symptoms in 38% of all weeks during the twelve months study period (11). Most URTIs have viral etiology and are self-limiting, yet some may progress to severe infections in need of treatment (24).

1.1.3.1 Common Cold (Nasopharyngitis)
The common cold is a heterogeneous group of mild URTIs of predominantly viral etiology (24). Symptoms include coryza, sore throat, and cough (25). The disease is estimated to cause 22 million days of absence from school annually in US children (26). The infections are in general self-limiting within two weeks and prolonged symptoms $\geq 2$ weeks are usually due to serial infections (27). No curative treatment is available, nevertheless, nasal cleaning and intranasal anticongestives have a role in the treatment of infants to facilitate feeding. A placebo-controlled randomized trial reported a role of honey in alleviating nocturnal coughing and sleep difficulties (28). There is poor evidence for cough medicines and mucolytics (24).

1.1.3.2 Pharyngotonsillitis
*Streptococcus pyogenes*, or Group A streptococci (GAS) accounts for approximately 37% of all pharyngotonsillitis episodes in children, however, less common in children below two years (29,30). Viral and bacterial aetiology cannot be accurately distinguished by clinical examination (31). Testing for GAS in children should be performed according to the Centor criteria (32). A rare but fearsome immunological complication to GAS pharyngotonsillitis is rheumatic fever, where destruction of the heart valves can lead to permanent heart failure. Antibiotic treatment seems to reduce the risk of the complication but the number needed to treat for one to benefit is very high in high-income countries (33).

Both chronic and acute pharyngotonsillitis is associated with a high grade of virus detection yet anaerobic bacteria such as *Fusobacterium necrophorum* are increasingly being recognized as underreported causes in children (34,35). Anaerobic infection should be a differential diagnosis in adolescents due to the risk of thrombophlebitis and septic embolization (*Lemierre syndrome*) (36). Mononucleosis or kissing disease is mainly caused by Epstein-Barr virus but is also associated with some other herpesvirus infections (37). The disease is characterized by tonsillar enlargement, moderate-to-high fever, petechiae of the palate, bilateral lymphadenopathy and splenomegaly (37). The disease has a long incubation period and is primarily seen in teenagers. Restriction of physical activity should be recommended for 3-4 weeks after the infection due to an increased risk of splenic rupture (37).

1.1.3.3 Acute Otitis Media
Acute otitis media is a usually self-limiting infection of the middle ear. Complication such as mastoiditis and labyrinthisis are very rare (38). The disease has traditionally been considered a bacterial infection but the role of respiratory viruses is increasingly being recognized (12). Current guidelines from the American Academy of Pediatrics recommend observation with close follow-up for non-severe (mild otalgia $\leq 48$h, fever $< 39^\circ$C) unilateral otitis in children $> 6$ months whereas antibiotic treatment with amoxicillin is recommended in complicated cases (38). In Sweden, antibiotic treatment with phenoxymethylpenicillin is recommended to children $< 1$ year, adolescents and in complicated cases whereas observation with follow-up is recommended children 1-12 years with uncomplicated disease (39).

1.1.3.4 Viral Croup (Laryngotracheitis)
Viral croup is a mild self-limiting infection in the larynx, sometimes involving areas of the lower respiratory tract. The disease mainly affects children between six months and three years of age (40). Classical symptoms are difficulties breathing, barky cough and stridor due to
inflammation of the mucous membrane of the larynx. Worsening is commonly seen during the night-time hours, which has been suggested to be due to low levels of anti-inflammatory endogenous serum cortisol (40). The duration of the disease is short, usually <48 hours and hospitalization is seldom needed. Parainfluenza virus is the most common cause of viral croup, but several respiratory viruses have been associated with the disease (40). Treatment with steroids is recommended in moderately severe cases whereas inhalation of epinephrine and oxygen treatment is indicated only in severe cases (40).

1.1.3.5 Other Upper Respiratory Tract Infections
Owing to successful immunization programs, the lethal upper respiratory tract infections epiglottitis (primarily caused by Haemophilus influenzae type B) and diphtheria i.e. “true croup” (caused by Corynebacterium diphteriae) are rarely seen in vaccinated children. A decline in the incidence of sinusitis has been seen following the pneumococcal conjugate vaccine (41).

1.1.4 Lower Respiratory Tract Infections
An estimated 703,000 deaths can be attributed to lower respiratory tract infections (LRTI) in children under five years annually (42).

1.1.4.1 Asthma and Wheezing
Asthma is a heterogenic chronic disease of the respiratory tract that is characterized by hyperreactivity of the immune system. The disease can be IgE-mediated (allergic asthma) or not (non-allergic asthma). Wheezing or sibilant rhonchi refers to a characteristic high-pitched whistling sound heard upon lung auscultation in children with asthma as well as in some viral infections. Although asthma is not an infectious disease, the acute disease is commonly triggered by viral infections and can mimic respiratory infections such as wheezy bronchitis. Moreover, treatment with the antibiotic azithromycin have shown to shorten the length of asthma-like episodes in children, which suggests a role of bacteria in the acute disease (43). However, it could also be the result of an anti-inflammatory effect of macrolides that has previously been observed (44).

Exposure to a large diversity of bacteria during childhood has been shown to be protective against asthma, which is commonly referred to as the hygiene hypothesis (45). In contrast, respiratory virus infections early in life have been associated with later development of asthma (46). It is not fully understood whether virus-induced wheezing is causing asthma or merely a first symptom of an underlying susceptibility (47).

1.1.4.2 Bronchiolitis
Bronchiolitis is a viral infection of the small airways in the lower respiratory tract. Acute inflammation with increased mucus production results in symptoms of coryza, cough, and low-grade fever frequently progressing into severe breathing difficulties (tachypnea, nasal flaring, use of accessory muscles). Diagnosis is based on a characteristic wheezing upon pulmonary auscultation and x-ray is not needed in uncomplicated cases (48). Bronchiolitis is usually restricted to children below one year of age whereas the diagnoses of wheezy bronchitis, reactive airway disease or non-allergic asthma are used for wheezing non-asthmatic episodes in older
The most common causative agents are respiratory syncytial virus, metapneumovirus and rhinovirus (48). Supportive care including nutrition and supplemental oxygen (in children with oxyhaemoglobin saturation ≤90%) are sometimes necessary. Inhalation therapy with hypertonic saline might alleviate symptoms whereas inhalation of epinephrine and salbutamol are no longer recommended due to lack of evidence (48,51). Neither do corticosteroids or chest physiotherapy have a role in the treatment (48,51).

1.1.4.3 Pneumonia

Pneumonia is an infection in the lung parenchyma characterized by high fever, tachypnea, indrawings, productive cough and lethargy. Complications include empyema or parapneumonic effusion. The disease is commonly divided into nosocomial i.e. hospital-acquired and community-acquired pneumonia (CAP). The incidence of childhood CAP is currently decreasing owing to a globally improved nutritional status as well as to vaccination against the two major causing pathogens. A simplistic algorithm for diagnosing pneumonia was released in 1990 by the World Health Organization (WHO) that was later included in the International Management of Childhood Illnesses (IMCI) strategy (table 2). The IMCI algorithm is based on respiratory rate, chest-wall indrawings, and cough and has been criticized for low specificity as episodes of bronchiolitis, asthma and dehydration frequently are misclassified as CAP (52). A clinical diagnosis is sufficient for non-severe cases that are treated as outpatients whereas radiographic confirmation is recommended in hospitalized patients (18).

Table 2 – IMCI Clinical Definition of Childhood Pneumonia.

<table>
<thead>
<tr>
<th>Sign or symptom</th>
<th>Classification</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cough or difficulty in breathing with:</td>
<td>Severe pneumonia</td>
<td>- Admit to hospital.</td>
</tr>
<tr>
<td>§ Oxygen saturation &lt;90% or central cyanosis</td>
<td></td>
<td>- Give oxygen if saturation &lt;90%.</td>
</tr>
<tr>
<td>§ Severe respiratory distress (e.g. grunting, very severe chest indrawing)</td>
<td></td>
<td>- Manage airway as appropriate.</td>
</tr>
<tr>
<td>§ Signs of pneumonia with a general danger sign (inability to breastfeed or drink, lethargy or reduced level of consciousness, convulsions)</td>
<td></td>
<td>- Give recommended antibiotic.</td>
</tr>
<tr>
<td>§ Treat high fever if present.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fast breathing:</td>
<td>Pneumonia</td>
<td>- Home care.</td>
</tr>
<tr>
<td>- ≥50 breaths/min in a child aged 2-11 months</td>
<td></td>
<td>- Give appropriate antibiotic.</td>
</tr>
<tr>
<td>- ≥40 breaths/min in a child aged 1-5 years</td>
<td></td>
<td>- Advise the mother when to return immediately if symptoms of severe pneumonia.</td>
</tr>
<tr>
<td>Chest indrawing</td>
<td></td>
<td>- Follow up after 3 days.</td>
</tr>
<tr>
<td>No signs of pneumonia or severe pneumonia</td>
<td>No pneumonia: cough or cold</td>
<td>- Home care</td>
</tr>
<tr>
<td>- Soot the throat and relieve cough with safe remedy.</td>
<td></td>
<td>- Advise the mother when to return.</td>
</tr>
<tr>
<td>- Follow up after 5 days if not improving.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


It is hard to establish etiology in childhood CAP. Bacterial blood cultures have limited sensitivity, respiratory specimens from the lungs are hard to acquire and specimens from the upper respiratory tract are clouded with bacterial colonization (53). Moreover, urine antigen tests for bacteria are not recommended for diagnostic purposes in children due to low specificity (18). Accordingly, an etiologic agent is rarely identified in children with CAP and antibiotic treatment is mostly prescribed empirically (16). Estimates of etiology of childhood pneumonia are largely varying (figure 3). During the 70s and 80s, several etiologic studies with invasive lung aspiration sampling were performed in low-income countries by the Board on Science and Technology for International Development. These studies identified bacterial causes in the majority of cases, primarily *S. pneumoniae* and *H. influenzae* and also identified the clinical signs associated with CAP that were later implemented in the IMCI guidelines (54). Viral causes were insufficiently studied due to diagnostic limitations. Later etiologic studies, mostly performed on upper respiratory specimens, have indicated an important role of respiratory viruses (55).
During the 1990s, vaccination against *H. influenza* type B vaccination was introduced, and since 2000, pneumococcal conjugate vaccines targeting certain serotypes of *S. pneumoniae* have gradually been introduced globally (57). This has likely contributed to the overall decline in CAP incidence seen in both low- and high-income countries (41). It is also hypothesized that, by targeting the two major bacterial causes, the relative proportion of viral etiology will increase (53). For long there has been a belief that viral infections pave the way for secondary bacterial infections. Yet increasing evidence point toward a role of viruses as sole causative agents of CAP (55,56,58,59).

A randomized placebo-controlled trial of non-severe CAP (clinical diagnosis) in Pakistan showed similar rates of treatment failure for placebo and amoxicillin suggesting that a large proportion of the study subjects had viral disease and hence did not benefit from antibiotic treatment (60). In contrast, *S. pneumoniae* was identified in 91% of lung aspirates of unvaccinated Gambian children with severe CAP, of which >75% were serotypes covered by current pneumococcal conjugate vaccines (61).

It is complicated to distinguish between viral and bacterial CAP and although commonly used, CRP is of limited use as certain viruses are associated with high CRP-levels (18). Parapneumonic effusion i.e. empyema is indicative of bacterial etiology with *S. pneumoniae* being the most common causative agent in unvaccinated settings (62). However, it is likely that the relative proportion of other bacteria such as *Staphylococcus aureus* will increase as etiologic agents in empyema given that invasive serotypes of *S. pneumoniae* are targeted by vaccines.

There are contrasting data on the role of atypical bacteria, such as *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae*, in childhood CAP, particularly in children ≤5 years (63,64). Current diagnostic tests for *M. pneumoniae* are inconclusive (65).

Swedish treatment guidelines for childhood CAP are focused solely on coverage of *S. pneumoniae* and penicillin V is recommended as first-line drug (66). The Infectious Disease Society of America recommends amoxicillin as first-line treatment in outpatients, whereas ampicillin or penicillin G are recommended for most hospitalized patients (18). This slightly extends the antimicrobial spectrum by adding additional coverage of most *H. influenzae*. In contrast, routine coverage for *M. pneumoniae* and *C. pneumoniae* is not considered necessary and should be restricted to patients with high suspicion of atypical etiology (18). Oral amoxicillin has been reported to be equivalent to parenteral penicillin G (67). For some reason, there seems to be a widespread skepticism to the usage of penicillin G among pediatricians and broad-spectrum beta-lactam antibiotics such as cephalosporins are widely used in hospitalized children with CAP (68). Nevertheless, Williams *et al* reported no difference in outcome between treatment with penicillin/ampicillin and third generation cephalosporins in hospitalized children with CAP in a retrospective register-based study using propensity score-matching (68).

**Figure 3. Estimates of Community-Acquired Pneumonia Etiology in Children.**
Estimates by (a) Scott *et al* and b) Jain *et al* (16,56).
1.1.5 Infections in Immunosuppressed Children

Immunosuppression refers to an impaired function of any part of the immune system owing to a secondary environmental factor, in contrast to congenital or primary immune deficiencies. Neutropenia, defined as a neutrophil blood count of $<1\times10^9/L$, is a common condition in children receiving chemotherapy as treatment for malignancies (69). The lowest neutrophil blood count, i.e. nadir, usually occurs 7-10 days after the treatment (70). A neutrophil count $<0.5\times10^9/L$ is considered severe neutropenia, which highly increases the risk of acquiring severe bacterial infections (69). Moreover, radiation therapy and chemotherapy damage the epithelial barrier, which increases the risk of bacterial translocation from the intestines. Altogether, these different mechanisms put children under treatment for malignancies at high risk of severe blood stream infections, i.e. sepsis or septicemia. The signs of illness are often discrete owing to the immune suppression hampering the inflammatory response. Fever is sometimes the only objective symptom of septicemia. For these reasons, treatment with broad-spectrum antibiotic therapy is started immediately in children with neutropenia during febrile episodes. This strategy, together with an improved chemotherapy have contributed to a remarkable increase in 5-year survival in pediatric malignancies over the last decades (71). Nevertheless, long courses of broad-spectrum antibiotics negatively affect the children in many ways, such as disturbing the gut microbiota, and increasing the risk for hospital-acquired infections. Moreover, in the majority of cases, no causative agent can be found (72). As RTIs are common in children in general, it is likely that they also play a significant role in febrile episodes in children under treatment for malignancies. Accurate diagnostics to distinguish between harmless viral infections and life-threatening bacterial infections are needed to reduce the high antibiotic pressure in this group.

1.2 RESPIRATORY PATHOGENS

Viruses are small (20-300 nm) infectious agents completely dependent on the cellular machinery of other organisms to successfully replicate (73). They consist of a small genome sequence packed inside a protein shell sometimes surrounded by an envelope, a lipid membrane. Viruses infect most known organisms including plants, animals and other microorganisms. They are classified according to their genome into DNA-viruses and RNA-viruses. More than 300 different virus serotypes have been associated with respiratory disease in human (table 3), of these, several have been discovered during the last two decades owing to molecular-based methods with increased sensitivity (figure 4). The infectious focus of respiratory viruses is usually less distinct as compared to bacterial infections. Consequently, the distinction between viral URTI and LRTI is sometimes delicate.

Figure 4. Year of Discovery for Respiratory Viruses Pathogenic to Human.
<table>
<thead>
<tr>
<th>Virus species (family)</th>
<th>Structure</th>
<th>Subtypes</th>
<th>Incubation period (74)</th>
<th>Associated diseases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus (Adenoviridae)</td>
<td>DNA non-enveloped, icosahedral</td>
<td>52 serotypes within the groups A-G</td>
<td>4-6 days</td>
<td>Tonsillitis, common cold, pneumonia, gastroenteritis, influenza-like illness.</td>
</tr>
<tr>
<td>Bocavirus (Parvoviridae)</td>
<td>DNA non-enveloped, icosahedral</td>
<td>1-4</td>
<td>unknown</td>
<td>Common cold, bronchiolitis.</td>
</tr>
<tr>
<td>Coronavirus (Coronaviridae)</td>
<td>RNA+ enveloped, helical</td>
<td>NL63, OC43, HKU1, 229E, MERS, SARS</td>
<td>3-4 days</td>
<td>Common cold, SARS, MERS.</td>
</tr>
<tr>
<td>Enterovirus (Picornaviridae)</td>
<td>RNA+ non-enveloped, icosahedral</td>
<td>≥67 serotypes within the subgroups A-D</td>
<td>3-6 days</td>
<td>Common cold, meningitis, hand-foot and mouth disease, systemic disease.</td>
</tr>
<tr>
<td>Influenza virus (Orthomyxovirus)</td>
<td>RNA- enveloped, segmented</td>
<td>A-C</td>
<td>1-2 days</td>
<td>“Influenza”, encephalitis, gastroenteritis, myositis, pneumonia.</td>
</tr>
<tr>
<td>Metapneumovirus (Paramyxoviridae)</td>
<td>RNA- enveloped</td>
<td>A and B</td>
<td>3-6 days</td>
<td>Bronchiolitis, pneumonia, common cold.</td>
</tr>
<tr>
<td>Parainfluenza virus (Paramyxoviridae)</td>
<td>RNA- enveloped</td>
<td>1-4</td>
<td>2-3 days</td>
<td>Viral croup, common cold, bronchiolitis, otitis media, pneumonia.</td>
</tr>
<tr>
<td>Respiratory syncytial virus (Paramyxoviridae)</td>
<td>RNA- enveloped</td>
<td>A and B</td>
<td>4-5 days</td>
<td>Bronchiolitis, common cold, otitis media, pneumonia.</td>
</tr>
<tr>
<td>Rhinovirus (Picornaviridae)</td>
<td>RNA+ non-enveloped, icosahaedral</td>
<td>&gt;100 serotypes within the subgroups A-C</td>
<td>1-2 days</td>
<td>Common cold, bronchiolitis, (pneumonia).</td>
</tr>
</tbody>
</table>
1.2.1 DNA-Viruses

DNA-viruses are genetically stable viruses that usually cause a low-grade persistent infection (73). Some DNA viruses cause latent infections that persist for years. DNA-viruses replicate in the nucleus.

1.2.1.1 Adenovirus

Human adenovirus (HAdV) was isolated from adenoid and tonsil tissue in 1953 (75). It was initially considered to be the “virus of the common cold” yet today we know that the virus is capable of infecting several organ systems, the two most common being the respiratory and the gastrointestinal tract. Tissue tropism partly correlates with specific serotypes (76). The virus is largely genetically conserved with a low mutation rate, instead, it escapes the immune system by causing chronic low-virulent infection and a large proportion of asymptomatic children are positive for adenovirus in their upper-respiratory tract (55,75–80). In contrast, acute HAdV infection is characterized by sore throat, high-grade fever, malaise, runny nose, conjunctivitis and diarrhea (76). The virus is also associated with severe LRTI and has been identified in lung tissue of deceased children with CAP (81,82). HAdV is capable of eliciting a substantial inflammatory response, thus mimicking bacterial infection in terms of CRP levels and white blood cell counts (83). This is one of the reasons for the frequent use of HAdV vectors in vaccine development. Systemic HAdV infection causing multi-organ failure is a major concern in immunosuppressed patients following organ transplantation (76).

HAdV is an extremely stable virus, highly resistant to drying, proteases in the gastrointestinal tract and even to most detergents (76). The incubation period is 5–6 days and outbreaks are relatively common (74,84). An oral vaccine against HAdV type 4 and 7 is estimated to prevent 13,000 episodes of febrile illness in US military recruits annually but is currently not used in civilian populations (84). The antiviral drug cidofovir is used in stem-cell transipients with severe HAdV infection and novel treatment strategies are in the pipeline (76).

1.2.1.2 Bocavirus

Bocavirus (HBoV) is a small single-stranded DNA virus in the parvoviridae family that was discovered by researchers at Karolinska Institutet in 2005 (5). The virus has been associated with both URTI and LRTI but is predominately detected in combination with other respiratory viruses and is also frequently detected in asymptomatic children (13,55,85,86). Whether the virus is a true human pathogen or merely a bystander is debated (87). HBoV single infection with high titers has been reported in children with severe respiratory tract infection suggesting a causal relationship (88).

1.2.1.3 Other DNA-Viruses

Polyomaviruses WU and KI are increasingly being recognized as respiratory viruses pathogenic to human (89). Some viruses in the herpesviridae family can present with respiratory symptoms (90).
1.2.2 RNA-Viruses

In contrast to the genetically stable DNA-viruses, RNA-viruses evade the immune system by constantly mutating. This is maintained by the unstable nature of the RNA and the lack of proofreading systems of the replication product. RNA-positive viruses have a similar configuration as the human messenger RNA and injection of the genetic material into the cell is sufficient for establishing infection (73). These viruses are usually non-enveloped and highly resistant to detergents. In contrast, the RNA-negative viruses need to be converted by a non-human enzyme carried by the virion (RNA-dependent RNA polymerase) prior to replication. For this reason, all RNA-negative viruses are enveloped making them more sensitive to drying as well as to ethanol and detergents.

1.2.2.1 Coronavirus

Human coronavirus (HCoV) is an enveloped RNA-positive virus that was discovered in the early 1960s (91). Despite being enveloped, the virus is capable of enduring the extreme environment of the gastrointestinal tract. There are six known species including SARS and the recently discovered Middle East Respiratory Syndrome (MERS). HCoV infection has an incubation time of 3-4 days and, with the exception for SARS and MERS, associated with a mild respiratory disease. In contrast, the novel strains SARS and MERS are highly pathogenic and caused outbreaks of atypical CAP with significant mortality among health care workers during 2003 and 2014 (24,92).

1.2.2.2 Enterovirus

Enterovirus (EV) is a non-enveloped RNA-positive virus within the picornaviridae family closely related to rhinovirus. There are four different species known to cause disease in human: A, B, C and D (93). The disease pattern is widespread, ranging from mild common cold, hand, foot and mouth disease, pleuritis and gastroenteritis to severe encephalitis, meningitis and myocarditis (93,94). Several outbreaks of highly pathogenic enteroviruses have been reported including the EV-D68 outbreak in 2014 causing severe respiratory disease with significant mortality and the EV-A71 outbreak of hand, foot and mouth disease in 2016 with severe neurological complications (95,96). A vaccine against enterovirus 71 was recently evaluated in a Phase III trial in China with reportedly good protection against hand, foot and mouth disease as well as against neurological complications (97).

1.2.2.3 Influenza virus

Influenza virus is an enveloped segmented RNA-negative virus that was first recovered 1933 (98). As opposed to other RNA-viruses, influenza viruses replicate in the cell nucleus. There are three types of influenza virus causing disease in human (A, B and C). Influenza A is a zoonotic and highly pathogenic virus (99). Influenza B is associated with similar symptoms as type A but does not cause pandemics. The incidence of influenza B is currently increasing in Swedish children (100). Influenza C is less pathogenic and associated with mild respiratory disease (101).

Characteristic influenza symptoms are rapid onset of fever, malaise, joint pain and cough. Atypical presentation such as febrile seizures or gastroenteritis is common in children (102). Influenza has been associated with CAP both as a primary pathogen and by predisposing for secondary bacterial infection (101). A large study in the US reported positive bacterial blood
cultures in only 2% of children hospitalized with influenza (3% in the subgroup of influenza positive children with a discharge diagnosis of CAP) indicating that severe secondary bacterial infections are rare (101). Other complications include otitis media, myocarditis, dehydration and encephalitis.

The incubation period is 0-2 days and transmission is mainly airborne (74). Aerosol transmission is facilitated in cold and dry climate, which is one reason for the seasonal influenza epidemics during winter (103). The virus is rapidly cleared from the respiratory tract after recovery and detection in asymptomatic children is uncommon (56,58,59). Nevertheless, large-scale serologic studies have suggested that a significant proportion of influenza virus infections are asymptomatic (104). Immunity is acquired after infection, but the virus is constantly mutating (antigenic drift), which allows individuals to be reinfected every year (73). Genetic segment reassortment of genes encoding the proteins hemagglutinin and neuraminidase is unique for influenza A and can occur when two different strains co-infect the same cell (antigenic shift) (105). By remodeling these two surface proteins, the virus efficiently evades herd immunity. Such novel influenza strains have caused several devastating pandemics throughout history (table 4).

### Table 4 – Historical Influenza Epidemics and Pandemics

<table>
<thead>
<tr>
<th>Influenza A strain</th>
<th>Pandemic/epidemic (year)</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1N1</td>
<td>Spanish flu (1918-1920)</td>
<td>Pandemic spread. Associated with 20-100 million deaths globally.</td>
</tr>
<tr>
<td>H2N2</td>
<td>Asian flu (1957-1958)</td>
<td>Pandemic spread. Associated with 1-1.5 million deaths globally.</td>
</tr>
<tr>
<td>H3N2</td>
<td>Hong Kong flu (1968-1969)</td>
<td>Pandemic spread. Associated with 0.75-1 million deaths.</td>
</tr>
</tbody>
</table>

Two antiviral drugs are approved for use in children, the orally administered oseltamivir (Tamiflu*) and the inhaled drug zanamivir (Relenza*). Both act as neuraminidase inhibitors which prevents the virus at the release stage, i.e. as they detach from the infected cells. Treatment with first generation neuraminidase inhibitors has been shown to shorten the clinical course with approximately one day and the best effect is achieved if treatment is started ≤12 hours after onset of fever (107,108). Seasonal influenza vaccines are developed annually and recommended to all children ≤5 year by the WHO (109). The evidence for these recommendations is weak and only a few countries have implemented this, including the US, the UK and Finland. The European council recommend immunization of certain high-risk groups (110).
1.2.2.4 Metapneumovirus

Human metapneumovirus (hMPV) is an RNA-negative virus in the paramyxoviridae family that was first described in 2001, however, serological analyses indicated that the virus had been circulating in humans for at least half a century (3). There are two genotypes (A and B) with a similar clinical presentation (111). The virus is closely related to RSV and has been associated with URTI, bronchiolitis, and CAP (13,58,112–114). It attributes to significant morbidity in young previously healthy children (115). In immunocompromised children, hMPV might progress to severe LRTI particularly in hematopoietic stem cell transplantation recipients (116). The virus usually follows an epidemical pattern in temperate countries, increasing in winter and spring with a largely varying incidence between years (114). Supportive care with oxygen, parenteral fluids is sometimes necessary for severe cases (50). Ribavirin and intravenous immunoglobulin have been used experimentally against hMPV CAP (117). No vaccine is available, but there are several ongoing vaccine projects at a pre-clinical stage (118). Detection of hMPV in asymptomatic children by PCR is uncommon (115).

1.2.2.5 Parainfluenzavirus

Parainfluenzavirus (PIV) is an RNA-negative virus in the paramyxoviridae family, that was first described in 1956 (119). Four subtypes (1-4) cause disease in human. PIV1 and PIV2 are the two most common cause of viral croup accounting for approximately two-thirds of all cases (120,121). Other manifestations include common cold and bronchiolitis. The virus has also been associated with CAP but the true causative role is not fully understood (55,56). No current treatment is available but antivirals specifically targeting PIV as well as vaccines are under development, although at an early experimental stage (122,123). Detection of PIVs in asymptomatic children is uncommon (13,124).

1.2.2.6 Respiratory Syncytial Virus

RSV is an enveloped RNA-negative virus in the family paramyxoviridae that was first discovered in 1956 (125). The virus follows an epidemic pattern in temperate countries and is a large burden on pediatric hospitals during the peak in the late winter months (126). For reasons not fully understood, the RSV epidemic usually have a regular biannual pattern in the Scandinavian countries, with an early intensive epidemic the first year followed by a late and less intensive epidemic the next year (127,128). The phenomenon has been suggested to be due to viral interference, but is likely also partly explained by herd immunity (127,128).

RSV infection mostly presents as a common cold infection, however, it is also the most common cause of bronchiolitis and viral CAP (126). Infancy, prematurity, chronic underlying conditions and immunosuppression are risk factors for severe disease and early RSV infection has been associated with the development of asthma (129). RSV CAP in immunosuppressed stem-cell recipients is associated with extensive mortality (130). Mutations in toll-like receptor 4 have been associated with severe disease (131). RSV bronchiolitis usually starts with 3-4 days of rhinorrhea and low-grade fever slowly progressing to severe breathing difficulties, increased mucus production, cough, wheezing, retractions and tachypnea (50). The characteristic clinical presentation is usually enough for a clinical diagnosis during the peak of the outbreak but should be confirmed by rapid antigen testing or PCR off-season and in atypical cases.

Pathogenesis is related to an excessive local immune response with massive infiltration of neutrophils in the lumen causing edema and bronchoconstriction and the virus evades the immune system via inhibition of type-1 interferon (132,133). Hospitalization and supportive
treatment with nutrition, oxygen supplementation, inhalation therapy with hypertonic saline and even mechanical ventilation or BiPAP/CPAP are necessary in some cases (134). Inhalation of epinephrine and salbutamol are no longer recommended by the American Academy of Pediatrics due to lack of evidence (48). Apnea is a rare complication to RSV infection where the pathogenesis is not fully understood (133). Protective antibodies are seldom acquired after infection and the virus is able to reinfec the same individual several time throughout life without undergoing antigenic change (135). Palivizumab (Synagis®) is a humanized monoclonal antibody directed against the F-protein of RSV that is used as prophylaxis in children at high risk for severe RSV infection. Current guidelines from the American Academy of Pediatrics recommend palivizumab during the first year in preterm children with chronic lung disease and in extremely preterm children (136). A randomized controlled trial on healthy preterm infants reported a significant lower number of wheezing episodes during the first year of life in children treated with palivizumab as compared to the placebo group (46).

After some major drawbacks including an early trial in the 1960s where a live-attenuated vaccine candidate caused lethal infection in two children, there are currently several ongoing vaccine projects ranging from early experimental studies to a Phase III clinical trial (Novavax®) (133,137). Maternal immunization is an alternative strategy to immunizing the children, as maternal antibodies transfer through the placenta and infants are at highest risk for severe infection (133).

1.2.2.7 Rhinovirus
Human rhinovirus (RV) is a non-enveloped RNA-positive virus that was discovered in 1956 (138). The genome resembles the human messenger RNA. There are more than 100 different serotypes described, divided into three groups: A, B and the recently discovered C (139). RV is the most common cause of URTI in both children and adults (24). Infection usually present as a mild, self-limiting “common cold” with runny nose, cough, rarely persisting more than 1-2 weeks (73). Immunity is evaded by frequent mutations and a large number of serotypes, hence preschool children commonly acquire serial RV infections during the winter season. RV-A and RV-C have been reported to be more pathogenic as compared to RV-B, but the difference does not appear to be extensive (140–142). Wheezing is a common symptom in toddlers and asthmatic children with RV infection, but the causal relationship between RV and asthma is debated (143). Certain single nucleotide polymorphisms have been linked to increased risk of RV wheezing indicating a genetic rather than environmental relationship (144).

RV is detected throughout the year, in the northern hemisphere the highest levels are observed in fall, preceding the yearly RSV and influenza epidemics (145). The non-enveloped structure makes the virus stable and resistant to drying as well as to many detergents. The virus is transmitted through fomites, i.e. contaminated objects such as hands but also spread as aerosols (73). RV replicates more robustly in epithelial cells at low temperatures, partly due to decreased antiviral activity of the innate immune system, which lends some support to the historical belief that low temperature causes common cold (146).

The novel antiviral pleconaril has shown to moderately alleviate symptoms and shorten disease duration of RV infection in adults but is not approved for use in children (147). RV is commonly identified in children with severe RTIs such as influenza-like illness and CAP but the causal relationship is debated as the virus is frequently detected in asymptomatic children (27,80,124,148–150).
1.2.2.8 Other RNA-Viruses

Many viruses that cause systemic infection, such as measles and hantavirus, can present with respiratory symptoms but are not further discussed in this thesis (90).

1.2.3 Gram-Positive Bacteria

Gram-positive bacteria commonly reside in the skin, as well as in the respiratory tract. They have a thicker cell wall as compared to Gram-negative bacteria, which absorbs the legendary Gram-stain that is still frequently used in hospital laboratories for diagnostic purposes (73).

1.2.3.1 Streptococcus pneumoniae

*S. pneumoniae* or pneumococcus, is an extracellular Gram-positive diplococcus known to mankind since the late 19th century (73). It has several virulence factors including a polysaccharide capsule, pili and hydrolytic enzymes located in the cell wall (151). *S. pneumoniae* is the most common cause of bacterial CAP, septicemia and meningitis in children and attributes to approximately 800,000 deaths in children below 5 years annually (152). Typically, pneumococcal CAP is characterized by rapid onset of fever and shortness of breath; coughing is not always present as there are only few cough receptors in the alveoli (153). Colonization of the upper respiratory tract is common in children, seen in approximately 25-80%, with higher rates in low- and middle-income countries (59,9). Colonization of *S. pneumoniae* is maintained by adhesion to epithelial cells through certain cell-wall associated proteins, whereas the polysaccharide capsule surrounding the bacteria protects from phagocytosis (8). Acquisition is usually not associated with symptomatic infection but seems to be facilitated by certain viral infections (154). Passive smoking and maternal pneumococcal carriage are other factors associated with colonization (154,155). *S. pneumoniae* is mainly transmitted horizontally via droplets and aerosols (155).

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Serotypes covered</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCV7 (Prevenar 7*)</td>
<td>4, 6b, 9V, 14, 18C, 19F, 23F</td>
<td>conjugate vaccine</td>
</tr>
<tr>
<td>PCV10 (Synflorix*)</td>
<td>serotypes in PCV7 + 1, 5, 7F</td>
<td>conjugate vaccine</td>
</tr>
<tr>
<td>PCV13 (Prevenar 13*)</td>
<td>serotypes in PCV10 + 3, 6A, 19A</td>
<td>conjugate vaccine</td>
</tr>
<tr>
<td>PPSV23 (Pneumovax*)</td>
<td>serotypes in PCV10 + 2, 3, 8, 9N, 10A, 11A, 12F, 15B, 17F, 19A, 20, 22F, 33F</td>
<td>polysaccharide vaccine</td>
</tr>
</tbody>
</table>

Invasive pneumococcal disease (IPD) is defined as detection of *S. pneumoniae* in normally sterile anatomical locations such as blood and cerebrospinal fluid (155). As colonization is a prerequisite for invasive disease, reducing the pneumococcal carriage in children has been a major public health goal during the last decades (155). There are more than 90 different capsular serotypes of the bacteria described, some are highly associated with invasive disease (155). These have been targets for pneumococcal vaccines (table 5). Immunization with
pneumococcal conjugate vaccine (PCV) have been successfully introduced to most parts of the world during the last 10 years and contributed to a declining incidence of IPD, as well as of sinusitis and CAP (156). In 2015, there were n=23 laboratory confirmed cases of IPD in Swedish children <5 years, the most common serotypes being 3, 22F and 19A (157). In Sweden, the 13-valent vaccine, PCV13, is most widely used (given to approximately 56% of all Swedish children) followed by PCV10 that is provided in certain Swedish counties (157). Despite a decline in invasive pneumococcal serotypes associated with the vaccination, the carriage rate has remained constant due to replacement of non-vaccine types (9). The 23-valent polysaccharide vaccine (Pneumovax®) has shown to have limited immunogenicity in young children and is currently only recommended to children >2 years at high risk of infection (158). Notably, PCV10 uses Haemophilus influenzae Protein D as one of the conjugate proteins, which seems to provide some protection against non-typeable H. influenzae (159).

1.2.3.2 *Staphylococcus aureus*
*S. aureus* is a Gram-positive coccus that is a common colonizer of the skin as well as of the respiratory tract (160). Once penetrating the epithelial barriers, the bacteria is capable of causing severe infections including abscesses, necrotizing CAP and septicemia (161). Infants and neonates are at highest risk for invasive disease (161). The pathogen is also a common cause of nosocomial i.e. hospital-acquired pneumonia in children but the role of CAP seems to be limited (18). Meticillin-resistant *S. aureus* (MRSA) is a large burden on the health care system and outbreaks in neonatology units are common globally, although not yet in Sweden (162,163).

1.2.3.3 *Other Streptococci*
*S. pyogenes* or Group A streptococcus (GAS), is a Gram positive coccus associated with skin infections as well as predominantly upper RTIs including pharyngotonsillitis, sinusitis, acute otitis media and mastoiditis (164). It is also a rare cause of necrotizing CAP (165). In contrast to GAS skin infections that commonly progress to septicemia, the risk for invasive disease in GAS associated pharyngotonsillitis is low (164). Group B streptococci is the most common cause of neonatal septicemia (166). Group C and G streptococci are less common causes of pharyngotonsillitis (73).

1.2.4 *Gram-Negative Bacteria*
Gram-negative bacteria have a thinner, and more complex cell wall as compared to Gram-positives, and are, in general, less susceptible to penicillins (73).

1.2.4.1 *Haemophilus influenzae*
*H. influenzae* is a small Gram-negative coccobacillus that was believed to be the cause of influenza prior to the discovery of the influenza virus in 1933 (98). *H. influenzae* is associated with a wide range of infections in the respiratory tract. The most important virulence factor is the carbohydrate capsule, which aids in evading the immune system by preventing from opsonisation and phagocytosis (167). Classification of the bacteria can be made based on the capsular structure into six serotypes (A-F). An efficient conjugate vaccine against the highly invasive *H. influenzae* type B (Hib) is available and has almost eradicated Hib meningitis and
epiglottitis in high-income countries (168). Nevertheless, in the year 2000, Hib still attributed to an estimated 371,000 deaths in children <5 years due to insufficient vaccination coverage (169). Non-typeable *H. influenzae* are increasingly being recognized as important pathogens in children (167). *H. influenzae* was the second most common cause of CAP in the early lung-aspirate studies from the pre-Hib-vaccination era (170). The current prevalence of *H. influenzae* in childhood CAP is largely unknown. Invasive cases are rare in Swedish children, with n=12 laboratory confirmed cases in children 0-4 years in 2015 (157).

1.2.4.2 Other Gram-Negative Bacteria

*Moraxella catharralis* seems to be an infrequent cause of CAP in children (171). The anaerobic bacterium *Fusobacterium necrophorum* is a rare, but likely underdiagnosed, cause of unilateral pharyngotonsillitis (35).

1.2.5 Atypical Bacteria

Bacterial causes of CAP, other than *S. pneumoniae* and *H. influenzae*, have historically been referred to as atypical bacteria, given that they usually have a milder disease presentation and only respond to certain classes of antibiotics (73).

1.2.5.1 *Bordetella pertussis*

Whooping cough is an infection in the bronchi caused by the bacteria *Bordetella pertussis* and *Bordetella parapertussis*. The typical clinical presentation includes violent coughing attacks followed by characteristic whooping episodes and apnea. Recently, the bacteria have also been associated with CAP (59,172). Leukocytosis is a common laboratory finding (173). The bacteria are also infrequent findings in children with CAP and associated with poor outcome (59,172). Whooping cough can last for several weeks but children are usually well-appearing in between the attacks complicating the clinical diagnosis. Coughing is triggered by a bacterial toxin that can persist for a period of time after the bacteria have been cleared. Antibiotic treatment has limited effect on the clinical course but is commonly prescribed to limit the spread of the disease (174). Eleven deaths in previously healthy unvaccinated infants have occurred in Sweden between 2003-2016 and the incidence seems to be increasing again, albeit from low levels (figure 5) (175). The old whole-cell vaccine was removed from the immunization program in Sweden in 1979 and replaced by an acellular vaccine in 1996. Between these years, no vaccination against pertussis was carried and hence the current parental generation is largely unvaccinated (175). Induced sputum sampling seems to improve diagnostic sensitivity as compared to nasopharyngeal sampling (59,172).
1.2.5.2 *Legionella pneumophila*

*Legionella pneumophila* is an intracellular Gram-negative bacterium associated with severe respiratory and gastrointestinal disease (176). The name originates from a lethal outbreak of CAP in US military veterans and the disease is commonly referred to as *Legionnaires' disease* (177). The bacteria grow in fresh water and several outbreaks associated with public swimming baths and cooling towers have been reported (176).

1.2.5.3 *Mycoplasma pneumoniae*

*Mycoplasma pneumoniae* is a bacterium that lacks a cell wall, which makes it resistant to beta-lactam antibiotics (73). It is associated with atypical CAP, mainly in school-aged children (16). Extra-pulmonary symptoms such as headaches and arthralgia has been associated with the disease (153) as well as severe manifestation of mucositis and encephalitis (178,179). A prospective observational study by Spuesen et al assessed *M. pneumoniae* in children with RTI, as well as in asymptomatic hospital controls. They reported that detection of *M. pneumoniae* by culture, PCR and serology, i.e. current routine diagnostic tests, was inconclusive due to an equally high degree of positive results among the asymptomatic controls (65).

1.2.6 Co-Infections and Viral Interference

Every infection triggers an immune response in the host. It has been known for long that influenza infection is associated with an increased risk for bacterial CAP even after recovery, which was evident in 1919 during the Spanish flu (180). Studies on mice have indicated that this

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**Figure 5. Incidence of pertussis in Sweden.** Number of reported cases in Sweden. Data acquired from the Public Health Agency of Sweden.
could be mediated by a desensitization of TLR-5 ligands in the sentinel cells of the lung (181). Advancement in microbiological diagnostic methods have allowed for sensitive detections of multiple respiratory agents and viral co-infections are today frequent findings in children with RTI (13). However, the clinical significance of viral co-infections in RTI remains elusive (182).

Viral interference refers to the interaction between virus outbreaks and has been reported between RSV and seasonal influenza (183). It is not clear whether this interaction takes place on a biological level (i.e. competition of target cells, upregulation of innate immunity etc) or on an epidemiological level (spreading patterns or behavioral differences). It was suggested that high levels of circulating RV hampered the spreading of the influenza A(H1N1)pdm09 (H1N1) pandemic in Sweden in 2009 (184). High levels of RV preceding the pandemic were also reported in Norway and France (185,186). Moreover, a retrospective study from Australia assessing respiratory specimens at the hospital laboratory reported that infection with RV was associated with a decreased probability of co-detection of other viruses (187). It is not fully understood whether this can be explained by a true causal relationship or whether it was just due to secular trends.

1.2.7 Microbiological Diagnostic Methods

There are several approaches to diagnosing infections (table 6). Some are directly targeted at detecting the pathogen (nucleic acids or cellular components) whereas others focus on the host response to an infection (serology, biomarkers). An optimal diagnostic test should have a high sensitivity and a high specificity. Sensitivity refers to the ability to correctly identify all patients with the disease i.e. to minimize the number of false negatives. Specificity refers to the tests ability to correctly identify patients without the disease i.e. high specificity indicates a low number of false positives. More importantly, the turnaround time is crucial for a test to play a role in clinical decision-making (16). Historical methods with slow turnaround times such as virus isolation and immunofluorescence microscopy have been replaced by faster and more sensitive molecular-based methods such as real-time polymerase chain reaction (PCR) (2). Electron microscopy still plays a role in detecting emerging pathogens (188). The technique has the advantage of visualizing unknown pathogens as opposed to PCR where the primers and probes have to be designed in advance based on already known motifs of the targeted microbes. Most point-of-care rapid tests are based on antigen detection and are in general less sensitive than PCR since they lack an amplification step. Next-generation sequencing (NGS) refers to different sensitive whole genome sequencing techniques that are increasingly being used in metagenomics, i.e. characterization of the complete genetic material in a sample. This approach has been used to discover several novel viruses (3,5,189). A disadvantage of all molecular-based methods is that the detections might reflect nucleic acid fragments rather than viable pathogens. Indeed many respiratory viruses have been shown to be frequent findings in asymptomatic individuals (13,55,79,80,90,124,150,190). Serology has poor correlation to PCR for certain viruses such as HAdV but correlates better for influenza (191).

Bacterial culture is an old but widely used method for detecting bacteria. The method also allows determination of antimicrobial resistance. Some bacteria are hard to culture and can only be identified by molecular-based methods. Moreover, the sensitivity of pediatric blood cultures is low. PCR-analysis of blood has been reported to improve sensitivity for detection of S. pneumoniae as compared to conventional blood cultures without compromising specificity, but is currently not used in the clinic (192).
<table>
<thead>
<tr>
<th>Method</th>
<th>Application</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture</td>
<td>Widely used in clinic for common bacteria and fungi.</td>
<td>Allows estimation of antimicrobial resistance.</td>
</tr>
<tr>
<td>(Virus isolation)</td>
<td>(Research)</td>
<td>Slow turnaround time, not useful for several viruses.</td>
</tr>
<tr>
<td>Electron microscope</td>
<td>For research purposes and surveillance of emerging epidemics of unknown pathogens.</td>
<td>Useful for new unknown pathogens. Visualize the structure of the pathogen.</td>
</tr>
<tr>
<td>Immunofluorescence</td>
<td>Research</td>
<td>High turn-around time, user-dependent. Visualize the structure of the pathogen.</td>
</tr>
<tr>
<td>PCR</td>
<td>Widely used in clinic for atypical bacteria, viruses.</td>
<td>Sensitive method. Only detects fragment of the pathogen. Sensitive to mutations of primer and probe binding regions of the viruses. Cannot detect previously unknown pathogens.</td>
</tr>
<tr>
<td>Next-generation sequencing (NGS)</td>
<td>Research purposes</td>
<td>Sensitive method. Creates large amount of data. Hard to evaluate the clinical significance. Slow and expensive.</td>
</tr>
<tr>
<td>Antigen tests (immunochromatographic tests)</td>
<td>Rapid tests for influenza A, RSV, GAS etc.</td>
<td>Rapid method. Limited sensitivity.</td>
</tr>
<tr>
<td>Serology</td>
<td>Severe atypical infections. Research</td>
<td>Detects the antibody response of the host. Sensitive, but slow turnaround time. Limited specificity due to cross-reactivity.</td>
</tr>
<tr>
<td>Microarray analyses (biochip)</td>
<td>Future method?</td>
<td>Allows detection of a very large number of pathogens.</td>
</tr>
<tr>
<td>Mass spectrometry</td>
<td>Research</td>
<td>Can be combined with nucleic-acid amplification methods as in the commercial pathogen identification system IRIDICA®</td>
</tr>
</tbody>
</table>
1.3 EPIDEMIOLOGY

1.3.1 Study Designs

Epidemiology is the core science of public health, but epidemiological methods and critical thinking can apply to most sciences. In the field of epidemiology, the main aim is usually to study the association between different exposures with a predefined disease or outcome. Different study design can be used depending on the research question. Experimental studies, mainly randomized controlled trials, are considered to yield the highest degree of evidence but are seldom feasible to conduct due to practical and ethical restrictions. For that reason, observational studies are often the only feasible alternatives. The two most common types of observational studies are cohort studies and case-control studies. In a cohort study, a defined population is followed until they eventually develop the predefined outcome. In case-control studies a reverse approach is used, where cases, with the outcome of interest, and controls, without the outcome, are identified and then assessed with regard to exposure.

1.3.2 Chance and Random Error

In every comparative analysis, there is a possibility that any potentially observed difference between two groups for a certain statistic was there just by chance. There are several statistic methods to handle this depending on the nature of the data (continuous or categorical, dependent or independent etc). Usually, confidence interval and p-values are calculated to give an estimate of the precision of the statistic or the point estimate based on the variance in the observations. Confidence intervals are commonly set at 95% corresponding to a p-value of <0.05. This means that the p-values are considered significant if we can be ≥95% sure that the observed effect is not there just by chance (assuming there is no bias in the study). Multiple testing violates this assumption. If twenty different tests are run in a study, one should be statistically significant just by chance, which is referred to as type-I error. In contrast, type-II error is related to the risk of rejecting a true effect due to a non-significant p-value. This risk is associated with the confidence level but also dependent on the study size and power.

Despite efforts in the study design, most studies will have some degree of random error, such as measurement errors, typing mistakes etc. These might increase the variance and negatively affect the precision of the study.

1.3.3 Bias

If the errors in a study do not occur at random, they are considered systematic errors or bias. This is a much more serious concern and directly affect the validity of the study. Bias has been categorized into three major groups: selection bias, information bias and confounding. Selection bias refers to a systematic error in selecting study subjects. If study participants differ from non-participants in terms of exposure and outcome the estimated association will be skewed if the study population is compared to the general population. Accordingly, it is important in case-control studies that controls are selected to be representative of the cases. Information bias includes misclassification of study variables or outcomes, which can be non-differential or differential depending on whether the misclassification is related to another study variable or not. Confounding refers to the confusion of effects. A confounder is a factor
associated with both the exposure and the outcome. An example would be smoking if one were to study the association between alcohol consumption (exposure) and lung cancer (outcome). Given that smoking is a verified cause of lung cancer and that it is possible that smoking is more common in individuals with high alcohol consumption, the association between alcohol consumption and lung cancer would be confounded by smoking. Such a study would need to control for smoking not to overestimate the association between alcohol and lung cancer. There are statistical methods to handle confounding, yet residual, unmeasured or uncontrolled confounding are always concerns in observational studies.

1.3.4 Infectious Disease Epidemiology

Some concepts in infectious disease epidemiology are unique for the field and for that reason, worth some extra attention. When studying diseases with human-human transmission one has to be aware that a case can also be an exposure i.e. cases can give rise to secondary cases. The basic reproductive number, $R_0$, is a measure of the average number of persons infected by a case in a totally susceptible population. This is dependent on how contagious the pathogen is, but also on the route of transmission, the number of contacts and duration of the infectious period. The $R_0$ is in general enhanced if the incubation period is shorter than the latency period, i.e. the infected individuals are infectious before they develop symptoms. If $R_0 > 1$ there is a risk that the pathogen will cause an epidemic. The fact that some individuals are usually immune to a certain disease will also affect the spreading patterns. The immunity on a population level is referred to as herd immunity and is of great importance for preventing disease outbreaks. The level of required vaccination coverage for preventing outbreaks is related to the $R_0$ of the disease. B. pertussis has a high $R_0$ whereas influenza is less contagious, for that reason, a vaccination coverage of 50-76% is needed to prevent influenza outbreaks whereas B. pertussis requires a higher coverage, approximately 92-93% (193,194).
2 AIMS

The general objective of this thesis is to improve the current knowledge of the causative role of respiratory microbes, predominantly viruses, in children with severe RTI with the long-term goal to reduce unnecessary antibiotic use in this patient group. This is important both in the general pediatric population, as well as in vulnerable populations, such as children with malignancies, to improve future preventive and treatment measures. To reach this goal a number of basic concepts must be clarified as defined by a number of specific aims.

2.1 PRIMARY AIMS

- To assess respiratory viruses in children with influenza-like illness
- To define the viral etiology of acute respiratory illness in children
- To define the viral etiology of community-acquired pneumonia in children
- To investigate the role of respiratory viruses in febrile neutropenia in children

2.2 SECONDARY AIMS

- To assess viral interference and the clinical impact of viral co-infection
- To investigate the clinical significance of PCR-positivity for respiratory viruses
- To characterize the clinical presentation of specific respiratory virus infections
3 METHODS

The rationale behind the choice of study designs as well as a brief summary of the methods is described below. Detailed information can be found in the Materials & Methods sections of the respective articles.

3.1 STUDY POPULATIONS

An overview of the study populations is presented in figure 6. In Paper I, children <18 years with influenza-like illness (ILI) admitted to inpatient care at Astrid Lindgren Children’s Hospital during the H1N1 pandemic (July – December 2009) were retrospectively investigated. The policy of the hospital during the pandemic was to perform PCR-based influenza diagnostics on all children with suspicion of influenza infection. For this reason, the study provided a rather complete picture of severe respiratory infections among the childhood population in Northern Stockholm. To ascertain independency between the observations, only the first episode was included for children hospitalized multiple times during the study period.

Paper II and Paper III were two matched case-control studies. This particular study design was chosen as detection of respiratory viruses by PCR was increasingly reported in asymptomatic children questioning the clinical significance of PCR-positivity (80, 150). For this reason, we believed that representative controls would be of great importance for studies on RTIs in children. The two studies were not typical epidemiological case-control studies that are usually retrospective, register-based and designed to identify risk factors for rare diseases. Instead, the studies were predominantly prospective, the outcomes were rather common and the disease had a short induction period. Cases were prospectively enrolled at Sachs’ Children and Youth Hospital and Astrid Lindgren Children’s Hospital, Huddinge. Inclusion criteria were age <5 years and acute respiratory illness (ARI) defined as the presence respiratory symptoms (≥1 of runny nose, sore throat, earache, cough, sputum production or dyspnea) (Paper II) or age <5 years and CAP based on radiological findings (Paper III).

Cases could only be enrolled once in the independency reasons. Healthy controls were enrolled by two research nurses at twenty-three different children health centers in Stockholm county during visits for vaccination and routine clinical check-ups (9). Information on socioeconomic status, co-morbidities and immunization status were collected for all study subjects using a standardized questionnaire. Controls with reported respiratory symptoms ≤7 days were excluded in Paper II since they otherwise potentially would have fulfilled the inclusion criteria for the cases. This exclusion criterion was not applied in Paper III in line with the study design of the large upcoming PERCH study (195). A major factor in case-control studies is the standardized and equal handling of cases and controls. Only a minority of children with respiratory symptoms are in the early phase of developing CAP and many pathogens can cause both mild URTI and CAP. By excluding controls with respiratory symptoms, the control group would be less representative of the source population from where the cases arise. I.e. they would likely be “too healthy,” and hence biasing the results toward an overestimation of the association.
One (Paper II) or two (Paper III) controls were individually matched to cases on age (+/- 6 months) and calendar time (+/- 14 days). Intervals were expanded to age +/- 12 months and calendar time +/- 30 days if no eligible controls were found. Microbiological sampling and radiological examinations as ordered by the treating physician were retrospectively collected for all cases from the medical records. Study periods were September 2011 – January 2012 for Paper II whereas inclusion continued until March 2014 for Paper III. No sampling was performed during summer.

In Paper IV, children ≤18 years undergoing treatment for malignancies were included during episodes of febrile neutropenia (FN) at the childhood cancer unit Astrid Lindgren Children’s Hospital in Stockholm, Sweden during January 2013 and June 2014. We initially wanted to enroll asymptomatic controls with neutropenia in the study but this was not
practically feasible. Instead, a longitudinal design with follow-up samples was chosen. FN was defined as a body temperature of \( \geq 38.5^\circ C \) on one occasion or \( \geq 38.0^\circ C \) on two occasions at least 60 minutes apart, combined with an absolute neutrophil count of either \( 0.5 \times 10^9/L \) on one occasion or \( 1.0 \times 10^9/L \) with a decline to less than \( 0.5 \times 10^9/L \) over a subsequent 48-hour period. Patients could be enrolled multiple times if they experienced recurrent episodes of FN during the study period.

### 3.2 SAMPLING

In **Paper I**, all respiratory specimens routinely collected in the clinic were used in the analyses including naso- and oropharyngeal swabs/aspirates as well as tracheal and bronchoalveolar lavages from a minority of the patients. In **Paper II-IV** nasopharyngeal aspirate sampling was used for all study subjects.

### 3.3 MOLECULAR ANALYSES

#### 3.3.1 PCR-Analyses

PCR is a fast and sensitive method for detection of respiratory viruses. A drawback is that the method is susceptible to antigenic drift and also merely detect nucleic acid fragments rather than viable viruses. Samples were extracted and analyzed by PCR for 16 different viruses at Karolinska University Laboratory, Solna (196). Duplex PCRs were used for all virus except for RV, EV, HAdV and HBoV. Extraction controls (EV, HAdV, influenza A and RSV A and B) and PCR-controls for each virus were used. The method is best described as semi-quantitative given that it renders cycle-threshold (CT) values inversely related to the viral load. First, the PCR is developed to favor detection of a broad range of clones (by using wobble primers, etc.) rather than to perfectly fit a particular virus strain. Also, respiratory viruses, mainly RNA-viruses, have a high mutation rate, for this reason, the amplification will not be entirely logarithmic to allow for backward calculation to the original quantity. Moreover, the exact amount of the input sample was unknown and respiratory specimens are more sensitive to sampling bias than for instance blood. One way to overcome this would have been to analyze a house-keeping gene from respiratory epithelial cells as a reference, which was not done (197). Finally, the exact concentration of the PCR-controls was not known. Instead the CT-values were monitored and the run discarded if the CT-value deviated more than two standard deviations from the mean of previous runs. Due to cross-reactivity between RV and EV, some samples were further analyzed by an in-house PCR for EV (**Paper II-III**) (198).

#### 3.3.2 Genotyping

In **Paper IV**, samples positive for RV and EV were sequenced (VP4/VP2 region). This had several advantages. First, it made it possible to distinguish between EV and RV accurately. Second, specific RV genotypes could be determined. Finally, cases with repeatedly RV-positive samples could be further assessed to make a distinction between virus persistence and new infections.
3.4 STATISTICAL ANALYSES

Data were analyzed in Stata version 12 and R. Fisher exact test and chi-square test were used for comparisons of independent categorical data whereas Mann-Whitney U-test and student T-tests were used for independent continuous data as appropriate. A p-value <0.05 was considered significant. In Paper II-III, McNemar’s test, paired T-tests and Wilcoxon signed-rank test were used for matched group comparisons as appropriate. Moreover, multivariate conditional regression analyses were performed with group (case or control) as outcome variable and age, viral co-infections and socio-demographic parameters as predictors. In Paper III the unexpectedly high numbers of co-infections created numerous unique strata when adjusting for confounders resulting in large drifts of the odds ratios. For this reason, matching was broken and cases were instead group matched to controls on year (2011, 2012, 2013), season (Fall, Winter, Spring) and age (<1 year and ≥1 years) in the final analyses. In Paper IV, multiple episodes were recorded from some of the study subjects. Nevertheless, independence between the observations was assumed and ordinary non-parametric testing was performed, in line with previous studies in the field (199–201).

3.5 ETHICAL CONSIDERATIONS

All studies were approved by the Regional Ethical Review Board in Stockholm. In Paper I, consents could not be collected due to the retrospective study design. Nevertheless, only study variables relevant for the study were collected and results were only presented at a group level.

In Paper II-IV written consents were collected from the parents to the study subjects. To minimize the violation of integrity, each study subject was assigned a unique study ID and the study key was available only to the primary investigators. The sampling methods that were used in the studies are standard procedures that in many cases would have been performed regardless of the study. Children in Paper II-III were given a small gift after sampling, but this was not announced until enrollment was completed. We believe that the benefit regarding increased knowledge about severe viral respiratory tract infections outweighed the slight discomfort for the children due to additional sampling.
4 RESULTS AND DISCUSSION

4.1 VIRAL ETIOLOGY OF RESPIRATORY TRACT INFECTIONS

In Paper I and Paper II, the viral etiology was assessed in children with ILI and ARI, i.e. children with suspected viral respiratory tract infection. In clinical practice, viral respiratory infections are rarely diagnosed, partly due to the lack of antiviral treatments but also due to lack of knowledge of disease presentation of specific respiratory viruses as well as of the clinical significance of PCR-positivity. For those reasons, a positive test result (perhaps with the exception of influenza and RSV testing) rarely alter the clinical management or recommendations given to the patients. Nevertheless, despite the lack of antiviral treatment alternatives, there is a benefit in accurately diagnosing the viral infection as this would improve prediction of the clinical course and infectivity as well as decrease overuse of antibiotics (190). The inclusion criteria used in the studies were broad; both children with URTI and LRTI could be included since inclusion was based on clinical symptoms rather than on radiologically verified location of the infection.

4.1.1 Influenza-Like Illness (Paper I)

In Paper I, only hospitalized children were included and as much as 48.6% suffered from an underlying chronic disease, representing a more vulnerable population than the general children population of Stockholm. Six children died during the study period; all had severe underlying chronic conditions. One or more viruses were detected in 61.6% of the cases. RV was the most common virus (detected in 28.1%) followed by H1N1 (16.5%) and PIV (6.6%) (figure 7). RSV and hMPV were rare, detected in 2.4% and 0.4% of the cases respectively.

**Respiratory Viruses in Children with Influenza–Like Illness**

- Rhinovirus
- Influenza A (H1N1)
- Parainfluenza
- Bocavirus
- Adenovirus
- Coronavirus
- Enterovirus
- RSV
- Metapneumovirus

![Figure 7](image_url)

**Figure 7. Respiratory viruses in children ≤18 years with influenza-like illness.**
Data presented as proportion of positive patients. Abbreviations: RSV, respiratory syncytial virus.
Since the focus of the study was on H1N1, the study period included the Fall and the early Winter months (no H1N1 was detected after December 31, 2010). This partly explains the low numbers of RSV and hMPV, as these viruses, in the Northern hemisphere, usually circulate later during the respiratory season (145). The microbiological data from Paper I are somewhat hard to generalize to other settings as the swine flu pandemic struck countries differently depending on national immunization strategies, time of introduction and perhaps also depending on other local virus epidemics. Even in Europe, there was a high degree of heterogeneity in the number of cases (185,186,202). The study underscores the overlap in symptoms of different viral infections in the respiratory tract given that only a minority of the patients with ILI tested positive for H1N1. Descriptive data such as these, have a value regarding virus surveillance and to generate hypotheses. Nevertheless, a shortcoming of Paper I is the lack of controls as a reference group, which severely hampers the causal inference of the findings.

4.1.2 Acute Respiratory Illness (Paper II)

In Paper II, children with ARI were studied. The study population resembled the one in Paper I in that sense that the inclusion criteria were based on clinical symptoms. However, they represented a less severely ill population given that all patients in Paper I were hospitalized in contrast to merely 10% in Paper II. Thirty-percent of the children presented with fever, 33% had an increased respiratory rate and none died during the study period. They could best be described as having mild to moderately severe disease, representing the large proportion of patients seen at a pediatric emergency unit. In total, 151/209 (72.3%) cases tested positive for one or more respiratory virus. This is line with other similar studies with a reported detection rate of 72-75%, yet higher than in Paper I, likely owing to the age difference as viral infections are more common in young children (80,150). In line with Paper I, RV was the most commonly detected virus (detected in 47.9%) followed by HBoV, HAdV and PIV (figure 8). No influenza viruses were detected during the study period.

Figure 8. Respiratory viruses in children ≤5 years with acute respiratory illness. PCR data of respiratory viruses detected in cases with acute respiratory illness (pink) and controls (green). Data presented as the proportion of positive patients. Abbreviations: RSV, respiratory syncytial virus.
4.1.2.1 Case-Control Analysis
To be able to account for asymptomatic detection of some respiratory viruses, a case-control study design was used in Paper II with individually matched population-based asymptomatic controls. Of the controls, 35.4% tested positive for one or more virus. Conditional logistic regression analyses assessing the association between detection of specific viruses and ARI showed significant associations for PIV (odds ratio (OR) = 16.0; 95% confidence interval (CI): 2.1-120.6), RSV (OR=11.0; 95% CI: 1.4-85.2), hMPV (OR=5.0; 95% CI:1.1-22.8) HBoV (OR=4.4; 95% CI:2.0-10.1), and RV (OR=3.5 95% CI: 2.2-5.6). Population-attributable proportions were calculated showing that 39% of all episodes in the cases could be attributed to RV. Detection of EV, HAdV and HCoV were not significantly associated with ARI underscoring the importance of proper controls in studies of RTIs in children. The clinical significance of PCR-positivity will be further discussed below (Results section 4.2.1).

4.1.2.2 Potential Sources of Bias
A significant limitation in Paper II is the lack of complete bacterial data. Bacterial infection is an obvious potential confounder in the study given that it is associated with the outcome (several bacteria can cause respiratory illness) and potentially also with the exposure (virus-bacteria interactions have been reported and will be further discussed in the results section 4.2.3). Additional bacterial analyses such as paired serology of common respiratory bacteria would have been valuable. However, there is currently no reliable methods for diagnosing bacterial upper respiratory tract infections and even with the most thorough bacterial diagnostic workup we would still not be able to distinguish between children with true viral respectively bacterial infections accurately.

Selection of controls that are representative of the cases is crucial in case-control studies. In Paper II, controls were enrolled at 33 different child health units during routine visits and vaccination. As Sweden has a uniquely high vaccination coverage of the child immunization program (approximately 99.7%), we reasoned that this was a suitable site for enrollment of population-based controls (157). Nevertheless, certain subgroups within the community such as migrants and children in families that are skeptical to vaccines are less likely to visit the child health centers. As these children still would show up at the emergency unit in case of illness, this was a potential source of selection bias.

Further, controls with respiratory symptoms ≤7 days were excluded since they otherwise potentially would have fulfilled the case definition. The downside of this was a risk of introducing bias. Respiratory symptoms were common in the children and almost 30% eligible were excluded as controls for this reason. If we, by doing this, selected a “too healthy” control population that differed from the cases with regard to exposure or host factors (such as immunity) this could potentially have biased the results to an overestimated association between respiratory viruses and ARI. Moreover, there is a risk that the exclusion of children with respiratory symptoms increased the proportion of children with other infections such as gastroenteritis. Some respiratory viruses, mainly adenovirus and enterovirus are associated with both respiratory disease and gastrointestinal symptoms. Consequently, this approach could have led to an underestimated association with ARI for these viruses, which is somewhat supported by the fact that we failed to detect a positive association with ARI for HAdV.

Only one season was studied in Paper II, which is also a limitation when generalizing the findings to other settings as the incidence of many respiratory viruses is varying between seasons (203).
4.1.2.3 A Review of the Literature

How do the results from Paper II agree with other studies in the field? Numerous case series have been published reporting descriptive PCR data of children with respiratory tract infection; nevertheless, only a limited number of studies with a proper reference group have assessed this association. Table 7 provides a list of case-control studies and studies with longitudinal sampling investigating viral etiology in acute respiratory illness in children.

Table 7. Viruses Associated with Acute Respiratory Illness in Children from Longitudinal and Case-Control Studies using PCR

<table>
<thead>
<tr>
<th>Study</th>
<th>Country</th>
<th>EV</th>
<th>Flu</th>
<th>HAdV</th>
<th>HBoV</th>
<th>HCoV</th>
<th>hMPV</th>
<th>PIV</th>
<th>RSV</th>
<th>RV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kusel et al, 2007 (204)</td>
<td>Australia</td>
<td></td>
<td></td>
<td>x</td>
<td>n/a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>van der Zalm et al, 2009 (79)</td>
<td>The Netherlands</td>
<td>n/a</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Singleton et al, 2010 (205)</td>
<td>US</td>
<td>n/a</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jansen et al, 2011 (80)</td>
<td>The Netherlands</td>
<td></td>
<td></td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iwane et al, 2011 (140)</td>
<td>US</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>x</td>
</tr>
<tr>
<td>Rhedin et al, 2014 (13)</td>
<td>Sweden</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chonmaitree et al, 2015 (12)</td>
<td>US</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Byington et al, 2015 (11)</td>
<td>US</td>
<td></td>
<td></td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td>x</td>
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</tr>
</tbody>
</table>

“x” indicates a reported significant association with acute respiratory illness.
Abbreviations: n/a, not assessed

The data from Paper II to a large extent agree with other relevant studies in the field. Significant association with ARI have been reported for hMPV, PIV, RSV and RV in most studies (table 7). In contrast, the association with ARI for HBoV is debated (86). Although there is increasing evidence that the pathogen is capable of causing respiratory tract infection (88), current diagnostic method seems to be limited in diagnosing the pathogen, which will be further discussed below (Results section 4.2.1).
4.1.3 Community-Acquired Pneumonia (Paper III)

4.1.3.1 A Need for New Studies on CAP Etiology
In Paper III we specifically wanted to assess the role of viruses in childhood CAP for a number of reasons. First, the disease contributes to significant mortality and morbidity in children, second, CAP has traditionally been considered a bacterial disease and antibiotics is prescribed on wide indications but the two major bacterial causative agents have recently become the targets of vaccines, which has likely led to a relative increase of other etiologies including viruses (1,16). Given that the IMCI guidelines for clinical diagnosis of CAP have poor specificity we chose a stricter case definition based upon radiological findings (206). Cases with CAP were enrolled at two pediatric hospitals in Stockholm both at the emergency unit and at inpatient wards during three consecutive respiratory seasons.

A total of 121 cases were included during the study period. Of these, 72% presented with fever, 72% were tachypnoeic, 59% had chest indrawings, 76% were admitted to inpatient wards and 2% were transferred to a pediatric intensive care unit. None died during the study period. Surprisingly as much as 81% of the cases tested positive for one or more virus. This is remarkable given that 95% of the cases received treatment with antibiotics for a suspected bacterial infection. Did all these patients really have a bacterial disease or were some of these children in fact suffering from a misclassified viral infection?

![Respiratory Viruses in Children with Pneumonia](image)

**Figure 9. Respiratory viruses in children <5 years with community-acquired pneumonia.** PCR data of respiratory viruses detected in nasopharyngeal aspirates of cases with pneumonia (purple) and healthy controls (green). Data presented as proportion of positive patients. Abbreviations: RSV, respiratory syncytial virus.

4.1.3.2 Case-Control Analyses
To further assess the association between respiratory viruses and CAP, conditional logistic regression analysis was performed taking into account the viral detections among the controls. Indeed, certain viruses were commonly detected in controls, mainly RV, HBoV and HCoV once again underscoring the importance of proper controls in studies of respiratory infections in children (figure 9). However, RSV (OR=10.1, 95% CI: 4.8-21.2), hMPV (OR=6.5, 95% CI:
and influenza virus (OR=4.2, 95% CI: 1.2-14.5) were only sporadically detected in the controls and highly associated with CAP. A week association with CAP was also seen for HAdV (OR=2.1, 95% CI: 1.0-4.3) but the association was not significant when adjusting for potential confounders. RSV, influenza or hMPV were detected in 60% of all cases questioning whether all the children with allegedly bacterial CAP really had a bacterial disease.

4.1.3.3 A Review of The Literature

The high degree of virus detections in childhood CAP found in Paper III, is supported by a prospective US study by Jain et al on >2000 hospitalized children with radiological CAP (56). They reported that viral etiology was likely in as much as 66% of all cases (figure 3b) (56). They reported similar detection rates of RSV (28% in all subjects, 37% in children below 5 years versus 32% in our study), hMPV (13% in all subjects, 15% in children below 5 years vs 23% in our study) and influenza (7% in both studies). They also sampled >500 hospital-based controls where all viruses except for RV (detected in 17%) were infrequent findings (≤3%), but no formal case-control analysis was performed.

Table 8. Viruses Associated with Pneumonia in Case-Control Studies using PCR

<table>
<thead>
<tr>
<th>Study</th>
<th>Country</th>
<th>EV</th>
<th>Flu</th>
<th>HAdV</th>
<th>HBoV</th>
<th>HCoV</th>
<th>hMPV</th>
<th>PIV</th>
<th>RSV</th>
<th>RV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fry/Dare et al, 2007 (207–209)</td>
<td>Thailand</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>x</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Berkley et al, 2010 (55)</td>
<td>Kenya</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>x</td>
<td>n/a</td>
</tr>
<tr>
<td>Wolf et al, 2010 (210)</td>
<td>Israel</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>x</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Mathisen et al, 2010 (124)</td>
<td>Nepal</td>
<td>x</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>n/a</td>
</tr>
<tr>
<td>Feikin et al, 2013 (211)</td>
<td>Kenya</td>
<td>x</td>
<td>n/a</td>
<td>n/a</td>
<td>x</td>
<td>n/a</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Yoshida et al, 2013 (149)</td>
<td>Vietnam</td>
<td>n/a</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhedin et al, 2015 (212)</td>
<td>Sweden</td>
<td>x</td>
<td>(x)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Self et al, 2015 (63)</td>
<td>US</td>
<td>x</td>
<td></td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Zar et al, 2016 (59)</td>
<td>South Africa</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spichak et al, 2016 (213)</td>
<td>Russia</td>
<td>x</td>
<td>x</td>
<td></td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PERCH (preliminary) (214)</td>
<td>Multi-center</td>
<td>x</td>
<td></td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

“x” indicates a significant association with community-acquired pneumonia.

1 Not significant in adjusted analysis. 2 PERCH study centers: Bangladesh, The Gambia, Mali, Kenya, South Africa, Thailand and Zambia. Abbreviations: n/a, not assessed.
Several case-control studies assessing the role of viruses in CAP have recently been published (table 8). Yoshida et al compared viral findings in Vietnamese children with WHO clinical CAP and in community controls. They reported significant associations for influenza A (OR=8.3) and RSV (OR=21.91). Self et al, reported significant associations with radiologically confirmed CAP for RSV (adjusted OR=15.2), hMPV (aOR=10.4), PIV (aOR=2.3) and HCoV (aOR=3.2) from a prospective study on children with CAP and hospital-based asymptomatic controls in the US (63). Zar et al, reported, in line with our study, that RSV (OR=8.1) and Influenza (OR=4.1) were associated with WHO clinically defined CAP in a nested case-control study of 284 South African children (59). In contrast to our study, they also found significant associations for HAdV (OR=2.2), HBoV (OR=2.3), PIV (OR=2.0) and cytomegalovirus (OR=1.6) but did not find a significant association with CAP for hMPV. One reason could have been the less specific definition of CAP that was used, which likely resulted in misclassification of children with bronchiolitis and wheezy bronchitis. Indeed, they reported that only 20 of 80 performed chest radiographs showed infiltrates suggestive of CAP. Spichak et al, presented data from a case-control study on 56 Russian children with radiologically confirmed CAP (213). Despite the limited study size, they could report significant associations for RSV (OR=7.7), hMPV (OR=21.1), influenza (OR=16.4) and HAdV (OR=15.5). Finally, preliminary data from the PERCH study show significant associations for influenza, RSV, hMPV and PIV (214).

In view of these studies, the significant associations with CAP for influenza, RSV and hMPV seem to be uncontroversial (table 8). It is surprising that PIV was not associated with CAP in Paper III. Our study period covered three consecutive seasons and variations in epidemiology over time, which is common for many respiratory viruses, should have been captured. Nevertheless, PIVs seem to be rather infrequent findings in CAP and our PCR panel did not include PIV4. Hence, we cannot exclude type-2 error as an explanation for the absence of a detected association. It could also be a result of PCR escape due to antigenic drift since PIV is an RNA virus with a high mutation rate. Indeed PCR escape was reported to be the reason behind a declining incidence of Chlamydia trachomatis in Stockholm, Sweden and has also been reported for influenza A (215,216). Whole genome sequencing of selected samples from the study are planned, which will potentially solve this issue.

There is disagreement between the CAP studies regarding the role of HAdV (table 8). In Paper III, significant association with CAP was seen for HAdV in the crude estimates, but this association disappeared in the multivariate analyses. Again, type-2 error is a likely possibility as the virus has previously been reported to be associated with CAP (59,213). Nevertheless, it is questionable whether PCR-positivity alone should be considered sufficient for diagnosis given the extensive detection in asymptomatic children (59). Interestingly, none of the case-control studies have supported a role of picornaviruses in CAP, contrasting several previous case reports (90). Although detection of EV or RV with current PCRs seems to be of limited significance in childhood CAP, certain strains such as EV-D68 have been associated with severe respiratory disease and future PCRs should perhaps focus on selected serotypes. With the exception of Iwane et al, none of the studies above report data on serotypes, which hampers interpretation on the role of distinct picornavirus serotypes in CAP.

To conclude, influenza, RSV, hMPV and PIV have been associated with CAP in several studies whereas the role of HAdV, HBoV and HCoV is less clear. There is currently no evidence from case-control studies for a role of RV or EV in CAP.
4.1.3.4 Association or Causal Relationship?
Similar to the discussion regarding the findings from Paper II, potential sources of bias such as confounding need to be considered to correctly interpret the significant associations between respiratory viruses and CAP in Paper III. Again, bacterial infection is the most important confounder and this was something we could not adequately control for given that the bacterial diagnostic testing was incomplete. Induced sputum samples, blood cultures and paired serology are additional samples that could have added robustness to the results but were not feasible to obtain due to logistic and financial reasons. In the routine microbiological workup that was performed by the attending physicians, a bacterial cause could be confirmed in a very limited number of cases. Only one out of 29 (3%) performed blood cultures were positive (growing S. pneumoniae serotype 38), 2/26 (8%) patients tested positive for M. pneumoniae by PCR and 5/121 (4%) of the children had evidence of pleural effusion on the chest radiographs, which is also indicative of bacterial etiology. There is clearly a need for improved microbiological diagnostic tests to distinguish between bacterial and viral CAP.

Another important potential confounder is socio-economic status (SES) as low SES could be considered to be associated both with a higher exposure to respiratory viruses as well as to an increased risk of CAP (outcome). Despite our efforts to sample controls representative of the cases at 33 different child health centers, we observed significant socio-demographic differences between the groups. For instance, smoking was more common among the parents of the cases and they also had a significantly lower educational level as compared to the parents of the controls. Average purchase power based on the residential areas of all study subjects was obtained to compare the study subjects with the general population (217). The cases lived to a larger extent in areas with a lower average purchase power index as compared to the controls (index 1.16 vs 0.75, p<0.001). Moreover, the average purchase power of the control’s residential areas was higher as compared to the general population in Stockholm indicating that we selected a “too wealthy” control population. This could partly be explained by a lower participation rate (estimated to 50%) among the controls than among the cases (estimated to 90%) as parents with low socioeconomic status usually are less likely to participate in research studies. We tried to adjust for the differences in a multivariate logistic regression analysis by including smoking and parental education level as predictors, which did not significantly alter the results. Nevertheless, as we did not have individual data on purchase power we could not adequately control for SES and thus residual or uncontrolled confounding by SES possibly overestimating the observed associations is a limitation of the study.

To conclude, Paper III adds further evidence that influenza, RSV and hMPV are causally associated with CAP. However, owing to the limitations above, we cannot tell whether they were the sole causes of disease or merely paved the way for undiagnosed secondary bacterial infections.

4.1.3.5 Estimation of CAP Etiology
Is it possible to give an estimate of the proportion of bacterial, viral and mixed bacterial-viral infections in Paper III? In the introduction of this thesis, two different CAP etiology estimates by Scott et al and Jain et al were presented (figure 3). Jain et al considered PCR-positivity for any respiratory virus indicative of viral CAP in the absence of bacterial diagnosis (defined as bacteria detected in blood or pleural fluid) (56). This definition can be criticized for lack of specificity and likely overestimated the proportion of viral CAP. Based on the current literature, it would seem more reasonable to limit the definition of viral CAP to detection of influenza, RSV, hMPV and PIV (table 8). An interesting study by Virkki et al reported that CRP cut-offs of
>80g/l in children <2 years and >120g/l in children >2 years were reasonably specific for bacterial etiology (218). In contrast, CRP <20 g/l was indicative of viral etiology. Could these cut-offs perhaps aid in the definition of the less obvious bacterial or viral cases? Figure 10 shows the estimated etiology in the cases from Paper III using a definition based of laboratory findings and CRP cut-offs as described by Virkki et al. In this estimate, respiratory viruses are the most common causes (45%) followed by mixed viral-bacterial (29%) and bacterial etiology (14%).

![Pie chart showing estimated etiology](image)

**Figure 10. Estimation of CAP Etiology in Paper III.**

Etiology of n=121 children with radiographic community-acquired pneumonia based on microbiological findings and CRP as follows: *Viral CAP*: PCR positive for influenza, RSV, hMPV, PIV, HAdV or PCR positive for other respiratory viruses if CRP <20. *Bacterial CAP*: Positive blood/pleura culture or lobar consolidation/large dense infiltrate on chest x-ray or CRP >80g/l in children <2 years/ >120g/l in children >2 years. *Mixed viral-bacterial infection*: fulfilling definitions for both viral and bacterial CAP. *Atypical bacteria*: Not fulfilling any above AND positive *M. pneumoniae*.

### 4.1.4 Febrile Neutropenia (Paper IV)

#### 4.1.4.1 Rationale of the Study

Respiratory viral infections are common in immunocompetent children; it is thus likely that they also play a role in febrile episodes in immunosuppressed children. Previous studies have indicated that respiratory viruses are commonly detected in children with FN, but none of these have compared the findings to a reference group, which is important considering the high grade of asymptomatic detections of viruses in children (13). In Paper IV, the presence of respiratory viruses was investigated during episodes of FN in children under treatment for malignancies. A longitudinal study design with repeated sampling was used, as an alternative to the case-control design. With this strategy, the cases served as their own individual controls.

#### 4.1.4.2 Assessing the Role of Viruses

At least one respiratory virus was identified in 39 (45%) of the 87 episodes. Previous case series have reported a detection rate between 44-57% (72,201,219–221) (table 9). RV and HCoV were the most common microbiological findings detected in 24% and 8% respectively (figure 11). A relatively low number of presumably more pathogenic viruses was detected, such as RSV, hMPV, influenza and a parainfluenza. This is in agreement with the fact that the majority of cases had mild respiratory symptoms.
### Table 9. Viruses Detected by PCR in Children with Febrile Neutropenia

<table>
<thead>
<tr>
<th>Study</th>
<th>Country</th>
<th>Percentage of positives</th>
<th>Most common findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Koskenvuo et al, 2008 (201)</td>
<td>Finland</td>
<td>61/138 (44%)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Rhinovirus, RSV</td>
</tr>
<tr>
<td>Lindblom et al, 2010 (200)</td>
<td>Sweden</td>
<td>41/90 (46%)</td>
<td>Rhinovirus, adenovirus</td>
</tr>
<tr>
<td>Torres et al, 2012 (219)</td>
<td>Chile</td>
<td>190/331 (57%)</td>
<td>RSV, rhinovirus, parainfluenza virus</td>
</tr>
<tr>
<td>Suryadevara et al, 2012 (220)</td>
<td>US</td>
<td>26/50 (52%)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Rhinovirus, influenza</td>
</tr>
<tr>
<td>Söderman et al, 2016 (142)</td>
<td>Sweden</td>
<td>39/87 (45%)</td>
<td>Rhinovirus, coronavirus</td>
</tr>
<tr>
<td>Santolaya et al, 2017 (221)</td>
<td>Chile</td>
<td>441/951 (46%)</td>
<td>Rhinovirus, RSV, parainfluenza virus</td>
</tr>
</tbody>
</table>

<sup>1</sup>HCoV-HKU1/NL63 not assessed. RSV, PIV, HAdV, influenza detected by immunofluorescence assay.

<sup>2</sup>HBoV not assessed. Abbreviations: PCR, real-time polymerase chain reaction; RSV, respiratory syncytial virus.

A follow-up sample was obtained for 32 of the virus-positive episodes to give a better picture of whether the detected virus was the result of asymptomatic shedding or indicated symptomatic infection. In 25 episodes (78%) the virus was cleared at follow-up (median time of 28 days). Sequencing of repeatedly positive RV samples revealed different serotypes in two cases. Hence the true clearance was 27/32 (84%). Shedding time of respiratory viruses is of particular interest in immunosuppressed children for the decision regarding isolation and cohorting as viral respiratory infections can be lethal in this group (130). The study was not designed to assess shedding time of specific viruses accurately, yet only a minority of viruses persisted >4 weeks.

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**Figure 11. Respiratory viruses in children <5 years with febrile neutropenia.** PCR data of respiratory viruses detected in immunosuppressed children during episodes of febrile neutropenia (indigo) and at follow-up (green). Data presented as proportions. Abbreviations: RSV, respiratory syncytial virus.
Despite the longitudinal design, the lack of a proper control group complicates the interpretation of the viral findings. Indeed, as discussed above the two most frequent findings, HCoV and RV, are reported to be common in asymptomatic children (13). Asymptomatic detection of respiratory viruses has not been properly assessed in immunosuppressed children. It is plausible that HCoV and RV are more pathogenic in the absence of a proper antiviral immune response. Studies of asymptomatic immunosuppressed children are needed to further evaluate the significance of PCR-positivity for certain respiratory viruses in this group.

How, can the findings from Paper IV be interpreted? In contrast to Paper I-III, where the bacterial testing was rather incomplete, an extensive microbiological workup including repeated blood cultures was performed in the majority of study subjects in Paper IV, as this is part of the routine management of children with FN. Despite this, only eight (9%) episodes of septicemia were recorded (of these, a respiratory virus was also detected in three episodes). All of these received treatments for an underlying hematological malignancy. It is known that stem cell recipients are at high risk for septicemia, which needs to be considered in the assessment of these children (199).

In 43 (49%) episodes, no pathogen was identified. Pediatric blood cultures have limited sensitivity; hence some of the culture-negative patients might still have suffered from a bacterial infection. This complicates implementation of the findings into clinical practice. Further, given that septicemia is such a severe disease and that antibiotic treatment historically has saved many lives in this group, implementing changes in the management of children with FN is a delicate task. It is unrealistic to believe that rapid viral diagnostics will have a significant impact on the administration of empirical antibiotic treatment in children with FN. Nevertheless, an interesting randomized controlled trial from Chile assessing treatment strategies in children with FN was recently published by Santolaya et al (221). The study evaluated safety and efficacy of withholding antibiotic treatment after 48 hours in children with negative blood cultures that tested positive for respiratory viruses. They reported low and similar complication rates in both groups and reinstatement of antibiotics was necessary for only 4/84 (5%) of the children in the antibiotic withholding group. The South American study shows that it is possible to implement an algorithm for discontinuation of antibiotics in children with FN, based on findings of respiratory viruses. This approach has great potential to reduce the antibiotic pressure in these patients.
4.2 VIRAL DIAGNOSTICS FROM A CLINICAL PERSPECTIVE

4.2.1 Significance of PCR-Positivity

The clinical significance of PCR-positivity of viruses in upper respiratory specimens has been questioned due to frequent detection of several viruses in asymptomatic children (2). PCR is not a functional assay and positivity indicates the presence of short virus-associated motifs of nucleic acid rather than the presence of viable infective virions. Methodological advancements, as well as good laboratory practice (usage of separate rooms, well-ventilated hoods, internal positive and negative controls etc.), have decreased the number of false positive test results (2). Moreover, given the unstable nature of the RNA molecule, it is unlikely that viral RNA fragments would persist a longer period in the respiratory tract without replicating. Indeed, degradation of RNA viruses associated with storage and handling of respiratory samples is a big concern when conducting studies on respiratory viruses. Further, as viruses are intracellular particles and replication requires infection of human cells, some claim that PCR-positivity in asymptomatic children always should be considered indicative of active infection, albeit sometimes subclinical (12). Regarding DNA viruses, this is not as clear given that these viruses, in general, are much more stable. Persistence of HAdV and HBoV in the respiratory tract have been reported (222). From a clinical utility perspective, the most important thing to know about the PCR result is whether it detects the causative agent or not, no matter if the potentially false positive result was due to mucosal contamination or indicated a true albeit subclinical infection. In Paper II, respiratory viruses were detected in more than a third of the asymptomatic controls. However, no follow-up of the study subjects was conducted and hence we could not say whether the controls developed respiratory symptoms after enrolment. It is likely that some of the virus-positive controls were sampled in the early phase of an uprising infection.

4.2.1.1 Asymptomatic Virus Detections by PCR

To further evaluate asymptomatic detection of viruses in the respiratory tract of children a literature review was performed and data from n=25 studies reporting PCR data on asymptomatic children were compiled (figure 12). In line with the findings from Paper II-III, detection of hMPV, RSV, influenza, EV and PIV was rare in asymptomatic children detected in 0.6%, 1.3%, 1.5% and 1.8% and 2.3% respectively (figure 12). Interestingly, with the exception for EV, all these viruses are enveloped RNA-negative viruses that are known to be more unstable and sensitive to drying and detergents as compared to non-enveloped ones (73). For EV, the interpretation of the meta-analysis is hampered by the limited power, given that EVs are infrequently detected also in symptomatic children with respiratory disease. Further, most PCRs are limited at distinguishing between RV and EV.
HAdV (7.5%), HBoV (13.4%), HCoV (6.7%) and RV (27.4%) were frequently detected in asymptomatic children thus obscuring the clinical significance of a finding (figure 10). This is in agreement with the results from Paper II-III. Regarding RV, HAdV and HCoV, differential pathogenicity between serotypes could be one explanation for the high asymptomatic detection rate. In Paper IV, all RV were sequenced to determine serotype. Indeed, only RV-A and RV-C, were found in the cases, which have been reported to be more pathogenic as compared to RV-B (140,209). Due to financial reason, RVs were not sequenced in Paper II-III but it would indeed be interesting to know whether RV-B was more common in the controls as a result of low virulent, asymptomatic infection. In terms of HAdV, it was recently shown that HAdV-C accounts for the majority of incidental HAdV detection in culture negative children (246). Since we did not have data on HAdV serotypes we cannot exclude a significant association between HAdV and CAP for individual serotypes. Exclusion of HAdV-C from the conventional PCR panel could be one possible measure to improve the clinical significance of PCR-positivity for that virus. Regarding HCoV, the limited number of observations positive for HCoV in the meta-analysis did not allow for individual assessments of specific serotypes. Interestingly, HCoV-OC43 was only detected in cases in Paper II (n=4). HBoV, is a virus commonly detected together with other respiratory viruses and its pathogenicity has been diligently debated since its discovery (86). Although it is established that the virus is a real human pathogen, it seems to cause persistent infection in some individuals. This is similar to other viruses in the Parvoviridae family and complicates interpretation of PCR-positivity (88,247). A limitation in the majority of the studies included in the meta-analysis was the lack of follow-up of symptoms to exclude children who were in the early phase of an infection.
4.2.1.2 Viral Shedding Time

Byington et al performed a longitudinal study with collection of weekly nasal swabs, as well as symptom diaries, from US children (11). They reported that all viruses except HBoV and RV were cleared from the respiratory tract within four weeks from the start of the infection. A limitation to the study was that RV specimens were not genetically sequenced. In another longitudinal study by Loeffelholz et al, the RV-positive specimens were genotyped, which showed that persistence of RV >4 weeks was rare (<5%) (248).

4.2.1.3 Significance of Viral Load

Jansen et al reported that RV-positive children with RTI, had higher viral loads as compared to RV-positive asymptomatic children in a prospective case-control study and speculated whether a cut-off could aid in the interpretation of PCR (80). As discussed in the methods section, the PCR method used in Paper I-IV was not designed to assess viral load accurately. Nevertheless, the CT-value, i.e. the number of cycles needed to amplify the target RNA/DNA to a detectable level, is inversely related to the input quantity and can thus be considered a rough estimate of the viral load. CT-values in virus-positive cases and controls from Paper II-III were assessed (figure 13). The median CT-values of detected HAdV, hMPV, PIV, RSV and RV were significantly higher in controls as compared to cases indicating a lower viral load. Although the data points largely overlap, it suggests that high CT-values have limited clinical significance.

![Figure 13. CT-values of Virus-Positive Children.](image)

Figure 13. CT-values of Virus-Positive Children. Scatter plot presenting individual CT-values of virus-positive cases with respiratory tract infection (pink) and controls (green). Horizontal lines representing medians. Abbreviations: CT, cycle-threshold; HAdV, adenovirus; HBoV, bocavirus; HCoV, coronavirus; EV, enterovirus; Flu, influenza virus; hMPV, metapneumovirus; PCR, real-time polymerase chain reaction; PIV, parainfluenza virus; RSV, respiratory syncytial virus; RV, rhinovirus.
4.2.2 Viral Co-Infections and Disease Severity

The role of viral co-infections in terms of disease severity is debated (182). As the sensitivity and microbiologic spectrum of our diagnostic tests increase, an increasing number of potential pathogens will be detected (2). In Paper I, viral co-infections were investigated over time in children with ILI. The study period ranged from July to December 2009. The number of co-infections increased over time, both in all PCR positive patients and in the subgroup of influenza positive children, to peak in December (figure 14). This is in line with a study by Grady et al who reported that low age, daycare attendance and Winter season was independently associated with viral co-infection (249).

![Figure 14. Viral Co-Infections over Time. Number of cases with viral co-infections in hospitalized children with influenza-like illness during 2009.](image)

The increased probability of co-infections during Winter is likely explained by two different mechanisms. First, RTIs, in general, are relatively uncommon during the summer months but increase in frequency during fall, owing to the start of schools and daycare facilities as well as to the climate getting dryer (103). As several different viruses are circulating at the same time, children infected with one particular virus will remain at risk for acquiring new infections of other viruses during their symptomatic phase, i.e. will result in an increased risk of co-infections. Second, as discussed above, many respiratory viruses are shed for a period after recovery. An increase in the acquisition rate of new infections will hence result in an accumulation of viral remnants from previous infections i.e. increase the number of false positive detections. Although it is sometimes a delicate matter to distinguish between true infective agents and contamination/colonization, an increasing number of studies suggest that many RTIs in children are caused by multiple pathogens (59,213).
4.2.2.1 Co-Infections in Children with Influenza

In Paper I, the association between co-infection and disease severity was specifically assessed in children with H1N1, as contrasting results previously had been reported (250,251). We could not detect any significant differences between children with H1N1 single infection and H1N1 viral co-infection in terms of disease severity (ICU treatment, complications, length of stay). This could be due to type-2 error since the power of the study was limited but it is also likely that co-infections with different viruses have a varying effect on disease severity. In Sweden, the H1N1 pandemic terminated before the peak of the seasonal RSV epidemic and we only detected low numbers of RSV during the study period. In contrast, high mortality was reported in Brazil where the two epidemics largely overlapped (250). Indeed, the high morbidity associated with influenza/RSV co-infection was recently shown in a large US cohort study by Lim et al (252).

Viral co-infections were also assessed in Paper II, and multiple viruses were detected in 42/229 (20.1%) cases. Viral co-infection, in general, was associated with discharge diagnoses of CAP (p<0.01) and bronchiolitis (p=0.02) as well as with signs of severe disease including decreased oxyhemoglobin saturation (p=0.04), tachypnea (p<0.01) tachycardia (p=0.05) and fever (p=0.05). The most common virus combinations were HBoV/HRV, HAdV/HRV, and HBoV/RSV, but the study size did not allow any further assessment of specific virus-virus pairs associated with disease severity. To conclude, there is little evidence of a general increase in disease severity by viral co-infection, however certain combinations, such as influenza/RSV, have been associated with high morbidity.

4.2.2.2 Viral Co-Infections in Asymptomatic Children

The study design used in Paper II and Paper III allowed for assessment of co-infections also in asymptomatic children. In Paper II viral co-infections were detected in 11/229 (5.3%) asymptomatic control children. Remarkably, in Paper III, two previously healthy control children without reported respiratory symptoms had five different viruses detected (hMPV/PIV3/RV/HBoV/HCoV-OC43 and hMPV/RV/HBoV/HAdV/HCoV-HKU1 respectively). Both of these children were also positive for S. pneumoniae in nasopharyngeal bacterial cultures (serotype 35F). No follow-up was performed on the study subjects and hence, we cannot tell whether this was a consequence of an impaired antiviral defense or if it occurred by chance. It is possible that respiratory co-infections are overrepresented in children with humoral immune defects as a result of an impaired virus clearance. Indeed, many primary immune defects usually become manifest in this age. However, this was not supported by Paper IV, given that the proportion of viral co-infections (7%), as well as the viral shedding time in the immunosuppressed children, seemed to be similar to what has been reported for immunocompetent children.

4.2.3 Interference between Respiratory Pathogens

4.2.3.1 Interference between Rhinovirus and Influenza A(pdm09)

An improved understanding of interference between different virus outbreaks is essential for disease prevention, not least regarding influenza surveillance. The rapid decline in H1N1 cases seen in December 2009 is interesting since the seasonal influenza epidemics, in general, peak later during the winter season. In contrast, RV is usually detected throughout the year with a peak during fall/early winter. Could the high levels of circulating RV explain the slow spread
and rapid decline of H1N1? Naturally, several possible explanations are confounding this observation but let us examine the data from Paper I. Figure 15 shows the number of cases over time for the two viruses. It is clear that both RV and H1N1 circulated during the whole study period and in fact, the two curves seem to follow each other and are both peaking in November. Moreover, five (6% of all H1N1 positive children) patients had co-infection with RV and H1N1. This would rather speak against the suggested negative interaction by RV on the H1N1 pandemic.

![Detection of Rhinovirus and Influenza A H1N1(pdm09) in Children with Influenza-Like Illness](image)

**Figure 15. Detection of H1N1 and rhinovirus over time.** Number of positive cases in hospitalized children with influenza-like illness during 2009. Abbreviations: H1N1, influenza A H1N1(pdm09); RV, rhinovirus.

The study relied solely on microbiological data from hospitalized cases with ILI and no data were available on controls. This made it impossible to estimate the virus circulating in the society where the potential interference would have taken place and advanced interaction analyses could not be performed. Nevertheless, other studies have supported the theory of interference between respiratory pathogens. Cowling et al reported that children receiving seasonal influenza vaccine had an increased risk for infection from non-influenza viruses in a randomized controlled trial (253). Moreover, interference between flaviviruses has been reported. Antibodies against dengue virus seem to predispose for zika virus infection by antibody-dependent enhancement (254). In Paper III, HCoV and HBoV were negatively associated with CAP. Could this be suggestive of a protective effect of infection with these viruses? Significant negative associations with CAP for HCoV was also reported in the preliminary results from the large PERCH study (ref RSV16 O’Brien). One explanation to the findings could be that the antiviral immune response elicited in the cases with presumably viral CAP protected against co-infection with HCoV.
### 4.2.3.2 Interference between Viruses and Bacteria

Regarding viral-bacterial interaction, there are solid data on the existence of interference between *S. pneumoniae* and respiratory viruses from large-scale observational vaccine studies (255,256). Weinberger et al reported a significant decline in hospitalizations for RSV infection in the US following the introduction of PCV in the national child immunization program (257). Madhi et al reported a decreased number of hospitalized children with viral CAP in South Africa after the introduction of PCV (256). *H. influenzae* is another pathogen commonly detected together with respiratory viruses, but the importance of such viral-bacterial co-infections needs to be further assessed (249). Co-colonization of *S. pneumoniae* and *H. influenzae* has been reported in several cross-sectional studies and there is increasing evidence of positive interaction between the two bacteria (160). In contrast, colonization of *S. pneumoniae* seem to decrease the probability for carriage of *S. aureus*, which has led to concerns about potential unwanted side effects from the PCV immunization (160).

### 4.2.4 Clinical Presentation of Respiratory Viruses

Clinicians commonly use symptoms and signs identified in the physical examination in the assessment of children with respiratory infection. Nevertheless, disease presentation of specific respiratory viruses is not entirely understood. In **Paper I**, we found a plethora of different viruses in the 502 children with influenza-like illness during the H1N1 pandemic. One reason was likely a liberal admission policy due to fear of severe H1N1 infection leading to hospitalization of a heterogeneous group of patients, yet the study underscores the overlap in symptoms of different viral respiratory infections as all cases were clinically suspected of having influenza. A recent systematic review reported that there is a lack of evidence supporting agent-specific symptom patterns for most respiratory viruses (258). The exception was RSV where significant associations with indrawings, wheezing and crepitations were seen for the pathogen in the meta-analysis (258).

#### Table 10 – Symptoms Associated with Detection of Respiratory Viruses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Associated symptom/characteristic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus</td>
<td>hypoxia (13)</td>
</tr>
<tr>
<td>Bocavirus</td>
<td>tachypnea, tachycardia, cough (13)</td>
</tr>
<tr>
<td>Enterovirus</td>
<td>fever (30)</td>
</tr>
<tr>
<td>Metapneumovirus</td>
<td>tachypnea, hypoxia, tachycardia, fever (13)</td>
</tr>
<tr>
<td>Parainfluenza virus</td>
<td>viral croup diagnosis (13)</td>
</tr>
<tr>
<td>Respiratory syncytial virus</td>
<td>tachypnea, wheezing (13)</td>
</tr>
<tr>
<td></td>
<td>productive cough, fever, age &lt;2 years (30)</td>
</tr>
<tr>
<td>Rhinovirus</td>
<td>coryza (13)</td>
</tr>
<tr>
<td></td>
<td>blocked nose (30)</td>
</tr>
<tr>
<td></td>
<td>absence of fever (13,30)</td>
</tr>
</tbody>
</table>

Data acquired from Rhedin et al and Thornton et al (13,30).
In Paper II, clinical symptoms and discharge diagnoses associated with detection of specific viruses were investigated in the cases with ARI. We were able to reproduce the reported association between PIV and viral croup (121). Moreover, RV was associated with coryza and absence of fever, i.e. suggestive of mild URTI. In contrast, hMPV and HBoV were associated with signs of severe LRTI.

There are some limitations to the analyses in Paper II mentioned above. First, type-I-error is a possibility given the numerous tests performed. Indeed, when performing Holm’s correction for multiple testing, none of the reported associations remain significant (data not shown). Second, the presence of viral co-infections complicated the interpretation. For instance, the majority of children with HBoV were co-infected with other respiratory viruses and there was a tendency that HBoV single infected children had a less severe disease as compared to HBoV co-infected children. This suggests that some of the morbidity could partly be attributed to the co-infesting viruses. A large-scale British prospective study, that assessed symptoms associated with respiratory agents in children in the primary care, was recently published by Thornton et al (30). In line with the findings from Paper II, they reported significant associations with non-febrile illness and blocked nose for RV whereas RSV was associated with signs of severe disease (table 10).

To conclude, there is currently limited evidence for agent-specific symptoms for viruses other than RSV and the clinical presentation of respiratory viruses largely overlap.
5 CONCLUDING REMARKS

• Several different viruses are detected in children with influenza-like illness and viral co-infections is common in influenza-positive children, which needs to be accounted for when attributing morbidity to influenza.

• The majority of episodes of acute respiratory illness in children can be attributed to rhinovirus. Other viruses significantly associated with the disease are bocavirus, parainfluenza viruses, respiratory syncytial virus and metapneumovirus.

• Respiratory syncytial virus, metapneumovirus and influenza virus are highly associated with radiological community-acquired pneumonia in children and viral pneumonia is likely an underdiagnosed disease.

• Respiratory viruses are commonly detected in immunosuppressed children during episodes of febrile neutropenia and prolonged viral shedding is infrequently seen suggesting a causal relationship.

• Asymptomatic detection of respiratory viruses in upper respiratory specimens is not uncommon in children. Detection of adenovirus, bocavirus, coronavirus, enterovirus and rhinovirus by PCR needs to be interpreted with caution in children with respiratory tract infection.

• Viral co-infection, in general, is not associated with increased disease severity, but certain virus combinations might be, such as influenza/respiratory syncytial virus.

• Complex interactions seem to occur between several respiratory pathogens but this needs to be investigated further in large population based studies or in experimental designs.

• Some respiratory viruses are associated with specific symptoms such as respiratory syncytial virus with wheezing, but the disease presentation of respiratory viruses largely overlap.
6 FUTURE PROSPECTS

6.1 PREVENTION AND TREATMENT

More than eighty years have passed since the discovery of the first virus, yet remarkably few antivirals are approved for treatment of respiratory infections. In contrast to the area of bacteriology, where the discovery of penicillin completely changed the whole field of medicine, there are still no “wonder drugs” with broad-spectrum antiviral activity. New vaccines and antivirals targeting respiratory viruses have great potential to fight childhood mortality and morbidity. The need for antivirals is biggest in the context of immunosuppressed children, for instance, the mortality of viral CAP in stem-cell recipients is 25-45% (130).

RSV is an excellent vaccine candidate as the virus is estimated to cause 66 000-199 000 deaths annually (259). Implementing an immunization program usually increases the average age of disease acquisition in the given population. No vaccines have a 100% efficacy and even if they result in a decreased amount of circulating virus, they will usually not make the virus disappear completely. This means that the exposure among non-immunized will decrease whereas the average time to disease increases. This effect can sometimes be a problem, as certain infectious diseases have a more severe clinical course if caught in adulthood as compared to childhood. If the vaccine efficacy is not good enough, then implementing a vaccine against such a disease could do more harm than good. Nevertheless, regarding RSV, this age effect would only prove beneficial given that infants are the ones that are at highest risk for severe disease. Developing an RSV vaccine is currently prioritized by many fundraising organization including the WHO and the Bill & Melinda Gates Foundation. Unfortunately, eliciting a long-lasting immune response to RSV without side-effects has proven difficult. There are currently more than 60 vaccine candidates ranging from preclinical studies to late Phase III studies, yet a well-functioning vaccine is likely many years ahead (133). Vaccination of infants is particularly challenging owing to their immature adaptive immune system. An alternative strategy currently receiving much attention, is maternal immunization, i.e. vaccination of mothers during pregnancy. As antibodies are transfused to the baby continuously during pregnancy, vaccination of mothers prior to delivery would provide protection to the child during the first critical months where they are at highest risk for severe disease (260).

Another paramyxovirus that only recently has received increased attention is hMPV. The virus attributes to substantial morbidity in children not least in immunosuppressed individuals (115,261). In Paper III, 23% of Swedish children hospitalized with CAP tested positive for hMPV. Such epidemiological studies are necessary to raise the awareness among scientist and industry stakeholders that the virus should be a highly prioritized target for future antivirals. Vaccination could be another potential future intervention, not least in certain high-risk groups, but so far no vaccine projects have reached Phase I (262). Finally, for PIV a vaccine seems excessive, but given that the virus is associated with severe disease in immunosuppressed children, new antiviral treatments would certainly have a major role in this setting (130).

There are some other viruses outside the paramyxovirus family who are worth discussing. HAdV is virus indeed capable of causing severe disease and a safe vaccine is already available, however, currently mainly used in US military recruits. Vaccination of certain high-risk groups in the civilian population might be considered in the future.

Influenza virus attributes to significant morbidity and mortality globally and is sometimes the sole cause of death in previously healthy Swedish children (100). Seasonal vaccines are developed annually but currently only recommended to high-risk populations in Sweden. These
vaccines are both safe and effective and used in a broader population in many other countries. For instance, in the US all children are recommended a flu shot. It is likely that providing seasonal influenza vaccination to all Swedish children would prove cost-effective and decrease both mortality and morbidity in the general child population. Influenza is also the only virus where an active antiviral drug is available. The use of oseltamivir (Tamiflu\textsuperscript{®}) in children, however, is very limited in Sweden. Likely due to limited diagnostics, but also to some extent due to lack of national guidelines (263,264). Given the extensive morbidity attributed to influenza and the high safety of current influenza treatment/vaccines, an increased use of oseltamivir, as well as nationwide immunization with seasonal influenza vaccines, would likely prove beneficial for the general child population.

Regarding bacterial vaccines, the PCV has reduced the disease burden of \textit{S. pneumoniae} significantly. Moreover, bacterial vaccines have the potential to specifically target clones highly resistant to antibiotics, which will likely play an even more important role in the future. The down-side of protein conjugate vaccines, however, is that they tend to lead to the replacement of non-vaccine clones and hence do not impact the colonization rate (9). In contrast, pneumococcal whole-cell vaccines have the potential to target all serotypes and hence impact colonization. An attractive whole-cell vaccine candidate has recently advanced to Phase II (265).

Childhood obesity is an increasing global problem that was recently reported to be an independent risk factor for severe influenza infection (266). It is also known that several nutritional deficiencies have an adverse effect on the immune system and prolonged Vitamin D supplementation in children has shown promise in preventing RTIs (266,267). Interestingly, a pathogen-specific effect related to nutritional deficiencies was recently observed in selenium-deficient mice. A benign strain of coxsackievirus had an increased mutation rate when inoculated in selenium-deficient mice, which resulted in increased pathogenicity also to non-deficient mice (268). Indeed, fighting poverty remains an important task for improving the global child health.

Finally, a big issue in pediatrics is the lack of approved indications and data on optimal dosage for existing drugs. Since most Phase III trials are performed on adults, predominantly males, several existing treatments are currently used off-label in children. This is a problem as children have a different metabolism and usually require substantially higher drug concentrations as compared to adults. For example, case reports of dental staining as an adverse effect of tetracycline treatment during pregnancy have for long limited the use of doxycycline in children ≤8 years [169]. This has favored the use of antibiotics with broader antimicrobial spectrum such as parenteral cephalosporins. Recently, these recommendations have been debated and there seems to be a lack of evidence that doxycycline increases the risk of dental staining, as opposed to older tetracyclines that had a much higher affinity for calcium (269). To limit the unregulated use, optimize dosing and evaluate historical dogmas, there is a need for clinical trials assessing the effect of existing antimicrobial drugs in a pediatric population, particularly in infants and neonates.

### 6.2 Diagnostics

The clinical WHO guidelines for diagnosing CAP need to be revised as they lack specificity and result in extensive misclassification of non-bacterial diseases as CAP (270). Point-of-care lung ultrasound could be one readily available tool to increase specificity without causing additional discomfort for the child and is increasingly used in South America (271). There is also a need
for new diagnostic tests that more accurately distinguish between viral and bacterial disease. Liquid chromatography tandem mass-spectrometry is a powerful tool to assess the complete proteome in diagnostic specimens and have been used to identify new biomarkers for respiratory infections (272,273). Viral biomarkers such as myxovirus resistance protein A (MxA) and TNF-related apoptosis-inducing ligand (TRAIL) might be a valuable complement to current inflammatory biomarkers that are mostly focused on bacterial infections (274,275). Given the complexity of the immune system, it is likely that a panel of biomarkers will be needed to distinguish between viral and bacterial infections accurately. Powerful techniques for this is already available such as liquid chromatography tandem mass-spectrometry and transcriptional profiling techniques (272,276). Such transcriptomic and proteomic approaches will likely be used more frequently in the future in combination with NGS screening for microbes. Not least will it be useful in the intensive care unit setting. However, to really impact the management of RTIs and decrease the overuse of antibiotics, cheap, rapid tests with a short turnaround time are needed. Recombinase polymerase amplification (RPA) is a nucleic acid amplification method that is almost as sensitive as PCR but does not require thermal cycling. An RPA-based point-of-care test could combine the advantage of a high sensitivity with a short turn-around time (277). As the test reaction can be carried out at room temperature, it is a particularly appealing method for resource-limited settings where the need for new diagnostic tests is high (278).

From a public health perspective, it is also important to implement existing guidelines. An Indian study reported that only 13% of children with CAP were given guideline concordant treatment, underscoring the need for continuous education of health care workers (279). Further, the unregulated over the counter selling of antibiotics that exist in many countries needs to be dealt with. It is important to keep in mind, though, that lack of access to antibiotics also attributes to significant mortality (280).

A significant proportion of the cases with a presumably viral disease in Paper I-II did not test positive for any virus. NGS screening of respiratory samples will likely result in the discovery of additional respiratory viruses in the future. Moreover, metagenomics studies assessing the complete microbiome will be critical to improve our understanding of interactions between respiratory microbes.
7 POPULÄRVETENSKAPLIG SAMMANFATTNING

Luftvägsinfektion, främst lunginflammation, är den enskilt vanligaste dödsorsaken hos barn i världen. Luftvägsinfektioner orsakas av både virus och bakterier. Sedan 1940-talet har upptäckten av antibiotika revolutionerat behandlingsmöjligheterna mot bakterieinfektioner. Tyvärr har det frikostiga användandet av antibiotika lett till att många bakterier idag är motståndskraftiga mot behandling och det är därför av största vikt att begränsa användandet av antibiotika till de fall där behandlingen är nödvändig för att kunna bromsa denna utveckling. Vad gäller behandling av virusinfektioner finns idag ett mycket begränsat antal antivirala läkemedel och vacciner riktade mot luftvägsinfektioner. Medan bakterieinfektioners roll är väl studerad, är många frågor fortfarande obesvarade avseende virusorsakade luftvägsinfektioner. Detta trots att majoriteten av alla luftvägsinfektioner orsakas av virus.

Nya molekylärbaserade metoder har revolutionerat diagnostiken av virusinfektioner. Från att i hög grad ha klumpat ihop samtliga virusinfektioner under begreppet “virus” börjar vi nu sakta bilda oss en uppfattning om skillnader i sjukdoms- och -förlopp för enskilda virus. Denna avhandling utgör en vidare kartläggning av detta med målet att bidra till en bättre förståelse för virusorsakade luftvägsinfektioner hos barn.


Sammanfattningsvis har studierna bidragit till en bättre förståelse för enskilda luftvägsvirus roll vid svår luftvägssjukdom hos barn, visat att många förmodade bakteriella infektioner hos barn sannolikt orsakas av virus samt belyst för- och nackdelar med rådande rutindagnostik för luftvägshämvittningar. Förhoppningen är att dessa resultat kommer bidra till en minskad antibiotikaanvändning och ge incitament till att utveckla nya diagnostiska test, antivirala läkemedel och vacciner mot luftvägsvirus.
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