On the epidemiology, clinical presentation and transmission of respiratory viral infections

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Cover illustration: Långberget, Värmland, meets outdoor temperature and annual peaks of the seasonal flu. By Nicklas Sundell.

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“If we knew what we were doing,
it wouldn’t be called research”

Albert Einstein

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ABSTRACT

Respiratory viral infections encompass a large heterogenous group of pathogens that constitute a major burden of disease globally. The various routes of transmission including airborne spread make them difficult to control. The aim of this thesis was to investigate the epidemiology, clinical presentation and transmission of viral airborne pathogens and respiratory viruses affecting the airways. In **paper I** over 20 000 clinical airway samples, referred for the detection of respiratory viral pathogens over a period of 3 years, were collected retrospectively and analysed for seasonal variation and relationship with meteorological factors. **Paper II** was a prospective study analysing the prevalence of respiratory viruses, as detected by PCR in nasopharyngeal samples, in 444 adults asymptomatic of respiratory tract infection. In **paper III**, clinical and laboratory differences of naïve measles infection compared to breakthrough infection, with focus on the risk of onward transmission, were investigated, in a retrospective analysis of a measles outbreak in Gothenburg 2017/2018. In **paper IV** we prospectively collected airway samples for multiplex real-time PCR in 220 adults hospitalized at the Department of Infectious Diseases with lower respiratory tract infection across three consecutive winter seasons.

Conclusions: The incidence of influenza and several other respiratory viruses are strongly associated with low outdoor temperature and low absolute humidity. The onset of the annual influenza epidemic is preceded by a sudden drop in temperature below 0 °C in our region. The prevalence of respiratory viruses in asymptomatic adults is low (<5%), suggesting that a positive detection by PCR is likely of clinical relevance when symptoms of respiratory tract infection are present. Breakthrough measles infection can be identified by history of vaccination and the detection of IgG at rash onset, and onward transmission from these infections is unlikely due to low viral load and mild respiratory symptoms. Viral infections and viral/bacterial coinfections are a common cause of hospitalization in adults with LRTI. Viral infections may have pronounced symptoms at presentation making them difficult to discern from bacterial infections.

Keywords: respiratory viruses, measles, influenza, epidemiology, meteorological factors, real-time PCR, viral transmission, lower respiratory tract infection

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SAMMANFATTNING PÅ SVENSKA

Infektioner orsakade av luftvägsvirus är mycket vanligt förekommande hos människan och är alltjämt förenat med en betydande morbiditet och mortalitet globalt. De orsakas av en stor och heterogen grupp av patogener vars spridningsmönster är komplexa. Särskilt spridning av luftburna virus är svår att förebygga. Denna avhandling syftar till att fördjupa kunskapen om virus som drabbar luftvägarna genom studier av epidemiologiska, kliniska och diagnostiska aspekter samt faktorer som kan påverka spridningen. Avhandlingen baseras på fyra delarbeten. I **delarbete I** utfördes en retrospektiv genomgång av drygt 20 000 kliniska virusprover från nasofarynx-sekret, analyserade med multiplex realtids-PCR, under en tre-årsperiod. Studien visar att aktiviteten av influensavirus och flertalet andra luftvägsvirus är starkt vinterbetonade medan rhinovirus och enterovirus finns året runt. Prevalensen av framförallt influensavirus korrelerar starkt till låg utomhustemperatur och låg absolut luftfuktighet. Starten av den årliga säsongsinfluensan verkar, på våra breddgrader, årligen sammanfalla med att medeltemperaturen per vecka hastigt faller under 0 °C. **Delarbete II** syftade till att undersöka prevalensen av luftvägsvirus hos vuxna utan aktuella symtom på luftvägsinfektion. Detta gjordes i en prospektiv studie där 444 asymtomatiska vuxna provtogs i nasofarynx med efterföljande multiplex real-tids PCR. Vi fann en låg förekomst av luftvägsvirus i denna population (<5%) vilket antyder att detektion av dessa patogener hos vuxna med pågående luftvägssymtom har klinisk relevans. I **delarbete III** utfördes en retrospektiv genomgång av ett mässlings-utbrott i Göteborg 2017/2018 med syfte att studera kliniska och virologiska skillnader mellan naiv infektion och mässling hos individ med tidigare genomgången vaccination (genombrottsinfektion) samt undersöka risken för fortsatt smittspridning vid dessa två tillstånd. Vi fann att majoriteten av de bekräftade fallen under utbrottet var genombrottsinfektioner och de gav inte upphov till sekundärfall. Naiva infektioner hade signifikant högre virusmängd i nasofarynx samt oftare hosta jämfört med genombrotts-infektioner vilket kan förklara skillnaden i smittsamhet. Uppgifter om vaccinations-historik samt analys av IgG antikroppar vid utslagsdebut gör det möjligt att skilja naiva infektioner från genombrottsinfektioner, vilket har stor betydelse vid smittspårning. I **delarbete IV** genomfördes en prospektiv studie där 220 vuxna, inlagda på infektionskliniken med nedre luftvägsinfektion, provtogs i nasofarynx för multiplex real-tids PCR. Vi fann att virusinfektioner och virala/bakteriella co-infektioner var den dominerande orsaken till sjukhusvård i denna grupp. Patienter med rena virusinfektioner hade ofta uttalade symtom vilket gör det svårt särskilja mellan viral och bakteriell genes i tidigt skede.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. A four year seasonal survey of the relationship between outdoor climate and epidemiology of viral respiratory tract infections in a temperate climate.

Sundell N, Andersson LM, Brittain-Long R, Lindh M, Westin J

J Clin Virol. 2016;84:59-63

II. PCR detection of Respiratory Pathogens in Asymptomatic and Symptomatic Adults.

Sundell N, Andersson LM, Brittain-Long R, Sundvall PD, Alsiö Å, Lindh M, Gustavsson L, Westin J

J Clin Microbiology. 2019;57(1):716-718

III. Measles outbreak in Gothenburg urban area, Sweden, 2017 to 2018: low viral load in breakthrough infections.

Sundell N, Dotevall L, Sansone M, Andersson M, Lindh M, Wahlberg T, Tyrberg T, Westin J, Liljeqvist JÅ, Bergström T, Studahl M, Andersson LM

Euro Surveill. 2019;24(17):2-12

IV. Community-acquired lower respiratory tract infections in adults requiring hospitalization: clinical characteristics and outcome in four different etiological groups.

Sundell N, Gustavsson L, Andersson LM, Lindh M, Westin J

In manuscript

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ABBREVIATIONS

RTI	Respiratory tract infection
URTI	Upper respiratory tract infection
LRTI	Lower respiratory tract infection
CAP	Community acquired pneumonia
HA	Hemagglutinin
NA	Neuraminidase
IFA	Influenza A
IFB	Influenza B
RSV	Respiratory syncytial virus
HRV	Human rhinovirus
HCoV	Human coronavirus
PIV	Parainfluenza virus
HMPV	Human metapneumovirus
HBoV	Human bocavirus
HEV	Human enterovirus
HAdV	Human adenovirus
RPV	Rinderpest virus
SARS	Severe Acute Respiratory Syndrome
MERS-CoV	Middle East Respiratory Syndrome Coronavirus
COVID-19	Novel coronavirus 2019
PCR	Polymerase chain reaction
qPCR	Quantitative real-time PCR
NP	Nasopharyngeal

Ct	Cycle threshold
R_0	Basic reproduction number
AH	Absolute humidity
VP	Vapor pressure
RH	Relative humidity
SH	Specific humidity
NEWS	National early warning score
CRB-65	Confusion, breathing rate, blood pressure, age ≥65
FU	Follow up

1 INTRODUCTION

Infectious diseases have accompanied mankind since the dawn of civilization. Numerous infections thrived under the living conditions that were offered humans in the pre-modern era. Poverty, starvation and war were the ultimate collaborating partners for communicable diseases. In this world, without the advancements of modern medicine, continuous outbreaks of contagious diseases were a part of daily life. Epidemics of plague, smallpox, measles and influenza, to mention a few, effectively decimated the populations. Even though the origins and causes of these diseases were unknown at the time, and rather were believed to be evoked by the wrath of God (or Gods), the concepts of quarantine and isolation were introduced early on. Nowadays, thanks to improved living conditions and the achievements in medicine, mankind have the upper hand in the fight against microbes, at least from a historical perspective. Antibiotics and vaccines have been our most effective weapons in this war. Previously fatal bacterial infections have disappeared in to the shadows. Devastating viral infections have been eradicated or have become rarities. Although many challenges lay ahead, such as the growing problem with antibiotic resistance and vaccine hesitancy, we can at least say that in terms of infectious diseases, the world is a better place now than in the dark ages. Nevertheless, there is, among others, one important group of infections that remains a major burden of health globally. These are the respiratory tract infections (RTI) that constitute a large heterogenous group of infections caused by a diversity of microbes.

Figure 1. The people of Tournai bury victims of the Black Death, 1353. Miniature by Pierart dou Tielt. (Downloaded from public domain)

Particularly viral RTIs are a continuous health issue by being the most common infections affecting humans worldwide yet with very few effective treatments at hand other than those offering symptomatic relief. Also, this group of viruses include several highly contagious pathogens, of which some have the potential to become pandemic.

The human airways encompass many essential anatomic structures, from the nasal cavity all the way down to the alveolar region. As a consequence, there is a wide spectrum of infections affecting the airways caused by many different respiratory pathogens. The clinical presentation of these infections many times overlap and offer little help in distinguishing the causative pathogen. Although the majority of RTIs are caused by respiratory viruses there is still a challenge for clinicians to discern viral from bacterial infection. Antibiotic overuse in patients with RTI remains a problem.

For clinical and diagnostical purposes RTIs are often divided into upper and lower respiratory tract infections. Upper respiratory tract infections (URTI) are usually self-limiting and involves the nasal cavity, pharynx, larynx and the large airways. Viral pathogens like human rhinovirus (HRV) and human coronavirus (HCoV) are commonly associated with URTI. Lower respiratory tract infections (LRTI) mainly affect the smaller airways causing bronchitis and bronchiolitis but generally also include bacterial and viral pneumonia. LRTIs are annually accountable for approximately 3 million deaths worldwide placing it number 4 on the list of top 10 global causes of death in 2016 [1]. According to a recent systematic review the most common pathogens associated with global LRTI mortality are *Streptococcus pneumoniae*, influenza, respiratory syncytial virus (RSV) and *Haemophilus influenza type B* [2].

When considering that RTIs are among the leading causes of death in both children and adults globally, and there is an urgent need for a reduction of antibiotic overuse in patients with respiratory viral infections, it is essential to learn more about the epidemiology, clinical presentation and transmission of these pathogens. The advent of quantitative real-time PCR (qPCR) panels for the detection of respiratory viruses in airway samples have enabled early and accurate etiologic diagnosis of RTIs but also opened the door to new research within this field [3, 4].

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As will be discussed more thoroughly in this thesis, there are several transmission routes of respiratory viruses. Airborne transmission is one of them and this route is not only restricted to respiratory viruses such as influenza. Other important viral pathogens such as the measles virus are also transmitted by the airborne route. Although measles often is classified as an exanthematous viral disease it exhibits many of the properties that is typical for respiratory viruses in terms of pathophysiology, clinical presentation and transmission. Hence, this important pathogen will also be addressed in this thesis.

In the coming section the different respiratory pathogens will be discussed in more detail.

Table 1. *Diameter, structure and taxonomy of viral pathogens discussed in the thesis.*

Viral pathogen	Size in diameter	Enveloped	Nucleic acid	Family
Influenza A	~100 nm	YES	RNA	Orthomyxoviridae
Influenza B	~100 nm	YES	RNA	Orthomyxoviridae
Human rhinovirus	~30 nm	NO	RNA	Picornaviridae
Respiratory syncytial virus	~150 nm	YES	RNA	Pneumoviridae
Human coronavirus	~125 nm	YES	RNA	Coronaviridae
Parainfluenza virus	~200 nm	YES	RNA	Paramyxoviridae
Human metapneumovirus	~200 nm	YES	RNA	Paramyxoviridae
Human bocavirus	~20 nm	NO	DNA	Parvoviridae
Human enterovirus	~30 nm	NO	RNA	Picornaviridae
Adenovirus	~100 nm	NO	DNA	Adenoviridae
Morbilli virus	~150 nm	YES	RNA	Paramyxoviridae

1.1 RESPIRATORY VIRAL PATHOGENS

virology, epidemiology and clinical presentation

1.1.1 Influenza A

Descriptions of influenza-related symptoms date back as far as the 12-th century and there is historical evidence of recurrent epidemics from the 15th century onwards [5]. The discovery of the influenza A virus (IFA) lingered until 1932 when it was isolated in nasal secretions from a patient with ongoing respiratory symptoms [6]. We now know that IFA is an enveloped virus that contains eight negative sense single-stranded RNA segments. It is a member of the *Orthomyxoviridae* family which also includes the human pathogens influenza B and C as well as the more newly discovered influenza D that infects pigs and cattle [7].

The eight RNA segments constitute the IFA genome which encodes 11 proteins with hemagglutinin (HA) and neuraminidase (NA) being the most important in terms of pathogenesis and natural evolvement [8]. These two antigenic glycoproteins are found in the outer layer of the virus. HA binds to epithelial cells thereby allowing invasion of the host cell. NA functions as an enzyme and exhibit two important mechanisms. Firstly, it prevents aggregation of inhaled virus particles in the protective coat of mucus lining the respiratory tract by inhibiting viral glycoproteins to bind to sialic acid-containing molecules in the proximity. The viral attachment to the epithelial cells, enabled by HA, could otherwise be blocked. Secondly, NA is essential for the release of newly assembled virions from the host cell allowing continuous transmission through ongoing symptoms of the respiratory tract. To date, 16 hemagglutinins (H1-H16) and 9 neuraminidases (N1-N9) have been described giving rise to multiple combinations of possible IFA subtypes. H1-H3 and N1-N2 are so far the only antigenic glycoproteins that have been involved in the subtypes that infect humans. However, avian influenza viruses, such as H5N7 and H7N9, which are adapted to birds, have also caused human infections with comparatively high case-fatality rates. Fortunately, so far, avian influenza viruses have not yet acquired the necessary tools for effective human-to-human transmission but they are a definite concern for future pandemics.

IFA is genetically unstable and is constantly evolving. The lack of proof-reading during replication leads to small point mutations in the RNA segments. The genetic diversity that is derived from this process is known as antigenic drift and ensures the sustainment of susceptible hosts within the population. This is an important prerequisite for the recurring outbreaks of the circulating subtypes (H1N1 and H3N2) [9, 10]. Furthermore, recombination of RNA

segments and exchange of viral RNA between the eight segments within a single viral strain may also contribute to antigenic evolution. Most importantly however, reassortment of the segments, that may occur when multiple influenza virus strains infect the same cell, can lead to new combinations by the acquisition of novel types of HA and/or NA. This process, known as antigenic shift, are associated with a more dramatic impact on the genetic diversity of the virus with the production of progeny virions. Reassortment are fundamental for the rise of new influenza pandemics through the introduction of novel subtypes to which the population are immunologically naïve [11, 12]. Influenza viruses have varying affinity to different species. Pigs and birds are the main animal reservoirs. As a consequence, two different variants of influenza virus (for example an avian subtype and a human subtype) that simultaneously infects a host (for example a pig) may lead to a novel strain by reassortment. The emergence of novel highly pathogenic zoonotic strains that may cross the species barrier remains a global health concern [13, 14].

The shift of subtypes has led to several well-described pandemics over the past 150 years. In 1889 to1890 the so-called Russian flue circled the globe. The responsible subtype is still up for debate though. In 1918 the Spanish flu, caused by a H1N1 strain, emerged and was followed by a worldwide pandemic with an estimated 50 million deaths [15]. The 20th century saw another two antigen shifts with subsequent pandemics through the Asian influenza in 1957 (H2N2) and the Hong Kong influenza (H3N2) in 1968, although with lower mortality rates than that of the Spanish flu. In April 2009 a novel strain of H1N1 (referred to as Influenza A(H1N1)pdm09 or swine flu) emerged, with initial cases in Mexico, and eventually spread around the world, but fortunately with lower death rates than first anticipated. The seasonal flue has since comprised the subtypes H3N2 and Influenza A(H1N1)pdm09.

The seasonal pattern of IFA is well described. In the temperate climate of the Northern and Southern hemisphere, the annual epidemics usually have an abrupt onset during periods of cold weather. The peaks are strictly confined to the winter months and are generally seen during December to February in the north and in May to July in the south. However, the introduction of a novel subtype may alter the epidemiological pattern as seen with the swine flu in 2009. IFA is rarely detected during the summer months in the temperate zone and any cases during this period have likely contracted the infection during travelling. In the tropics and subtropics, the epidemics are less pronounced and sometimes exhibit a year-around activity with increased incidence during humid and rainy conditions [16]. Nevertheless, the reasons behind the seasonal variation of IFA remain enigmatic to a large extent and are still not fully

understood. The seasonal pattern of influenza in Sweden, over a period of 5 years, is displayed in Figure 2.

Compared to many of the other respiratory viral pathogens, IFA causes pronounced symptoms in an infected individual. After an incubation period of 24-48 hours there is an abrupt onset of high fever, malaise and myalgia. This is accompanied by a sore throat, dry cough and also occasionally conjunctivitis. In uncomplicated cases, symptoms resolve spontaneously within a week. However, complications to influenza are a common cause of hospitalization worldwide and include primary viral pneumonia, secondary bacterial pneumonia and other secondary bacterial infections in the airways. Other complications like myocarditis or CNS associated conditions, such as encephalitis, are sometimes seen, albeit less frequent.

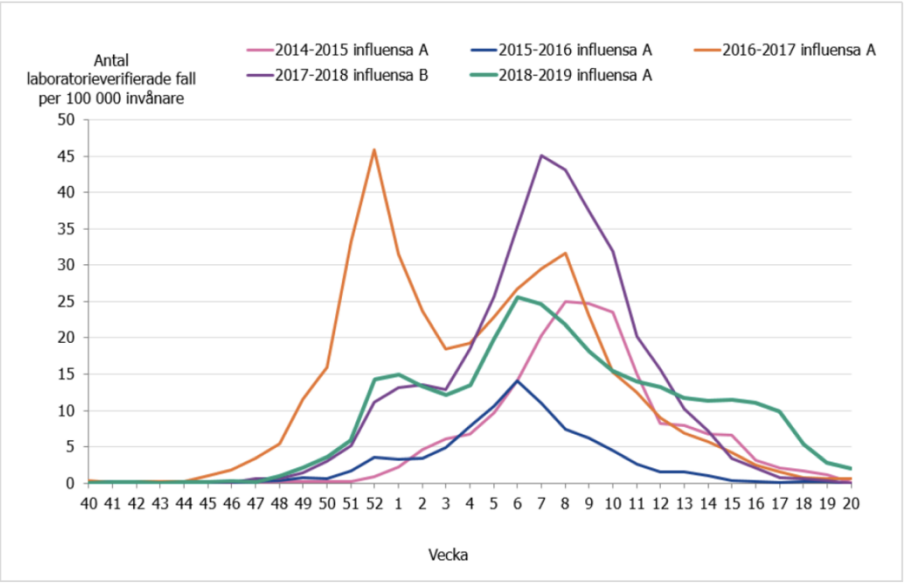

Figure 2. The weekly incidence of laboratory confirmed influenza cases in the elderly (>65 years of age) in Sweden during five winter seasons (2014-2019). Source: Public Health Agency of Sweden.

1.1.2 Influenza B

Influenza B (IFB) is, second to IFA, the most important member of the influenza virus family in terms of morbidity and mortality. The virus is also part of the *Orthomyxoviridae* family and is only known to infect humans and seals. IFB is, compared to IFA, a homogenous group of viruses that have diverged into two main lineages based on antigenically characteristics; the Yamagata lineage and the Victoria lineage [17]. Antigenic drift and reassortment occurs in IFB viruses and genetic diversity may explain the recurring epidemics [18]. Nevertheless, the restricted number of hosts may play an important role as to why the virus does not exhibit the same annual epidemic pattern as IFA.

In the Northern hemisphere IFB often appears biennially or with an interval of a few years and the epidemics are usually confined to the cold winter months. The symptoms resemble those of IFA and the infections cannot be distinguished from one another based on clinical evaluation alone. In general, the elderly and the immunocompromised may be more prone to develop complications to IFB infections. For example, during 2017-2018 flu season in Sweden, IFB was predominant among adults admitted to the ICU with laboratory confirmed influenza of whom almost 75% of the cases were >65 years of age or belonged to at-risk population [19].

1.1.3 Human rhinovirus

Human rhinovirus (HRV), a member of the *Picornaviridae* family, is a positive sense single-stranded RNA virus with a capsid enclosing the genome. Through translation, a total of 11 proteins can be produced of which VP1, VP2 and VP3 are important for the genetic diversity of the virus [20]. HRV is classified into three groups; HRV-A, HRV-B and HRV-C, with the latter being discovered more recently after the introduction of more sensitive molecular techniques [21]. Genomic sequencing has further revealed the detailed features of HRV serotypes opening a window for potential novel antivirals [22].

HRV causes the common cold and is responsible for 2-3 symptomatic RTIs in an adult per year. A higher frequency is observed in children, who also are considered to be the main reservoir of the virus [23, 24]. Interestingly, this pathogen does not exhibit the same seasonal variation as seen in other respiratory viruses. Instead HRV infections seem to occur all year-around but sometimes with an increased activity during spring, summer and fall [20]. The clinical symptoms in the immunocompetent individual typically involve a runny nose, sore throat and cough but rarely high fever. The infection is self-limiting and symptoms resolve within 5-7 days. However, in the immunocompromised host, HRV infections have been associated with

increased morbidity and mortality, especially in stem-cell transplanted patients [25-27]. Furthermore, HRV may cause asthma and COPD exacerbations and are therefore accountable for numerous health care visits and hospitalizations annually [28, 29]. However, the clinical relevance of HRV, when detected in adults with community acquired pneumoniae (CAP), is not fully understood.

1.1.4 Respiratory syncytial virus

RSV, first isolated in 1957, is a pathogen of global importance and with a significant disease burden, particularly in infants. It is a negative sense single-stranded RNA virus belonging to the *Pneumoviridae* family (within the *Orthopneumovirus* genera since 2016). Two major antigenic groups form subtype A and B. Different circulating genotypes within each subtype may partly explain the recurring seasonal outbreaks. Mutations, accumulating during RNA replication due to the lack of proofreading, may also contribute. However, compared to the eight segments of IFA, the non-segmented genome of RSV inhibits the capacity of reassortment and subsequently also antigen shifts.

RSV infections occur throughout the world and much like influenza the annual epidemics appear during wintertime with peaks in January to March in the north and May to July in the south. A more irregular pattern is often seen in the tropics with increasing incidence during rainy periods in general.

Although RSV may cause symptomatic RTI at all ages, the major clinical manifestation is LRTI in form of bronchiolitis in immunologically naïve infants. Globally RSV is still associated with a significant morbidity and mortality in this age group. A recent study estimated that RSV accounted for 7% of the global mortality in children (in the age of 1-12 months) making it, next to malaria, the second most common cause of death in this age group [30]. At the age of 2 years, most children have developed measurable levels of specific antibodies after previous infection. The presence of antibodies does not seem to prevent from re-infections but the clinical course is milder in older children and in adolescence. Nevertheless, healthy adults also develop LRTI occasionally. RSV infection in the elderly may be associated with more severe disease including bronchiolitis and CAP [31-33].

1.1.5 Human coronavirus

Human coronavirus (HCoV) is an enveloped positive sense non-segmented RNA virus that was first isolated more than 50 years ago [34]. The genome, which consists of a large RNA molecule, is encased by a nucleocapsid. The genome codes for four to five different proteins of which the S-protein, that protrudes through the encasement, is important for the formation of

neutralizing antibodies [35]. Four major genera form the coronavirus-group (alfa, beta, delta and gamma). The strains causing human respiratory infections include HCoV-NL63, HCoV-229E, HCoV-HKU1 and HCoV-OC43, and belong to the alfa and beta group. Coronaviruses in the delta and gamma group are primarily found in animals such as bats [36]. The last two decades have witnessed the emergence of three novel and highly pathogenic strains of coronaviruses; Severe Acute Respiratory Syndrome (SARS), Middle East Respiratory Syndrome Coronavirus (MERS-CoV) and novel coronavirus 2019 (COVID-19). All of them belong to the beta genera [37, 38]. Alarmingly high case-fatality rates have been reported for SARS and MERS-CoV. These zoonotic infections have sparked a global concern for new pandemics, but luckily there has been a limited spread from human to human. In January 2020 a novel coronavirus (now officially named COVID-2019) was found in a cluster of people suffering from an unknown pneumonia [39]. Initially, a seafood market in the city of Wuhan, China, seemed to be the common ground of exposure. As of yet the number of cases is steadily rising and available data have confirmed human-to-human transmission [40]. The exact routes of transmission are under debate at this early point. Although many details of COVID-19 are still waiting to be unravelled, the rapid increase of newly confirmed cases from day to day, with death-rates already surpassing the SARS outbreak in 2002-2003, have been enough to declare a global health emergency by WHO. Furthermore, the lack of antiviral treatment and available vaccines against HCoV are an obvious concern.

The four strains (HCoV-NL63, HCoV-229E, HCoV-HKU1 and HCoV-OC43) are commonly found in clinical specimens worldwide. They typically appear to be more frequent during the cold months but smaller peaks may occur more irregularly. Different circulating strains may contribute to the seasonal pattern as demonstrated in a nine-year survey of HCoV infections in children in Norway [41].

From a clinical point of view most HCoV infections typically resemble those of HRV, generating symptoms of a mild URTI with a runny nose, sore throat and cough. The infections are usually self-limiting but secondary bacterial infections in the upper and lower airways are sometimes seen. Immunocompromised individuals may be at risk of more severe disease. Re-infections seem to occur frequently, probably due to waning immunity after infection. Antigenic drift has also been proposed to contribute to the epidemiological pattern of HCoV [42].

1.1.6 Parainfluenza virus

Parainfluenza virus (PIV) was discovered in the 1950s and comprises four genetically and antigenically different types (PIV1-PIV4). They are all members of the *Paramyxoviridae* family which contains other important human pathogens such as the measles virus, mumps virus and human metapneumovirus. PIV 1 and 3 are associated with LRTI and they are members of the same genera, *Respirovirus*, while PIV 2 and 4 belong to the *Rubulavirus*. PIV is an enveloped negative sense single-stranded RNA virus and is not a strictly human pathogen also infecting a variety of animals. Similar to influenza viruses, the genome encodes the hemagglutinin-neuraminidase protein but antigen shift does not seem to occur. However, nucleotide substitution has been reported [43-45].

The seasonal appearance of PIV is probably dependent on the geographical location and the serotype. A Swedish study found PIV to be more active between April and June [46]. Others have reported that PIV 1 and PIV 2 tend to accelerate during fall in the temperate climate zone whereas PIV 3 may circulate within a region throughout the year with peaks that are more difficult to predict. PIV does not seem to display any clear seasonal pattern in the tropics however [47-49].

During childhood, PIV is associated with both URTI and LRTI. Croup is a common manifestation in children and familiar to many clinicians (and parents). The broad spectrum of respiratory symptoms caused by PIV makes it difficult to discern from other respiratory viruses. From a clinical point of view, it is important to recognize that PIV may cause severe respiratory disease with significant mortality in immunocompromised individuals, especially in patients with hematologic malignancies [45, 50].

Neutralizing antibodies seem to be serotype specific and offer little cross protection. Thus, re-infections occur throughout life. Cellular immunity is also important for the protection against PIV which could explain the severe illness that can be observed in patients with a more profound immunosuppression.

1.1.7 Human metapneumovirus

Human metapneumovirus (HMPV) is closely related to RSV but belongs to another genera (*Metapneumovirus* genera). It is a non-segmented negative sense RNA virus with two main disease-causing subtypes (A and B). It was first discovered in 2001 but has probably been circulating among humans for more than 100 years [51, 52]. Recombination as well as selection forces seem

to play an important role in the evolution of the virus but from a broader perspective it maintains a limited genetic diversity [53].

HMPV affects all age groups and may cause both URTI and LRTI. The virus may, just like RSV, cause bronchiolitis in infants as well as LRTI and pneumonia in children, sometimes requiring hospitalization. The incidence of HMPV infections is especially high in children and specific antibodies are measurable from early on in life [54]. Re-infections occur, plausibly due to waning immunity. Antigen drift has been described but does not seem to contribute to re-infections in the same extent as in influenza viruses. HMPV usually peaks during wintertime in the Northern hemisphere but outbreaks do not always coincide with similar disease-causing pathogens like RSV [55].

In healthy adults, HMPV generally causes a mild RTI including cough, nasal congestion and runny nose but rarely fever. The immunocompromised host and the elderly may be at risk for more severe disease and fatal cases have been reported [56-58].

1.1.8 Human bocavirus

Human bocavirus (HBoV), discovered in Sweden in 2005 by Allander et al., is a member of the *Parvoviridae* family [59]. It is a small enveloped single-stranded DNA virus. Since the identification of HBoV1 from human respiratory samples in 2005, an additional three genotypes have been isolated (HBoV2-HBoV4) from stool samples [60]. Bocavirus, especially HBoV1, has been associated with respiratory infections in general but more specifically bronchiolitis during infancy [61]. The pathogen has been detected in clinical samples worldwide and the high seroprevalence among young children indicates that HBoV infections occur already in early childhood. HBoV is rarely detected in adults and any infection is most likely restricted to milder respiratory symptoms. However, a recent report by Lee et al. highlights that HBoV can be associated with more severe respiratory infections among older adults regardless of immune status [62]. The seasonal trends of HBoV is not fully understood but epidemiological and clinical studies have reported an increased incidence from late winter to early summer [62, 63].

1.1.9 Human enterovirus

Human enteroviruses (HEV) are ubiquitous and more than 70 serotypes have been described. It is a non-enveloped single-stranded RNA virus and part of the *Picornaviridae* family. Clinically important members of the enterovirus family are polioviruses, coxsackievirus A and B, echoviruses and enterovirus 68-71. The vast number of serotypes result in a broad spectrum of infections encompassing everything from mild RTI and gastroenteritis to severe and

sometimes fatal disease due to meningoencephalitis and flaccid paralysis. From a global and historical perspective, polio has been the most feared HEV infection causing significant morbidity and mortality in the pre-vaccination era. Although poliovirus, which causes the devastating paralytic poliomyelitis, is targeted for global eradication, it remains a continuous health problem in a few countries such as Nigeria and Pakistan.

HEV is a common cause of RTI in all age groups and the incidence is usually peaking during summer and fall. The endemicity of enterovirus is generally due to a few circulating serotypes. The appearance of more pathogenic strains such as enterovirus D68 and A71 warrants the need for constant surveillance of circulating serotypes. Although antibodies against common enteroviruses are found in a high proportion of the population, the number of serotypes and the lack of cross-reacting immunity, especially in children, allow re-infections to occur [64, 65].

1.1.10 Human adenovirus

Human adenovirus (HAdV) is a clinically important virus with more than 60 serotypes described. It is a non-enveloped double-stranded DNA virus. Based on antigenic features, the number of serotypes is further classified into subgroups A-G [66, 67]. Neutralizing antibodies are serotype-specific with little cross-reactive protection but the cell-mediated immune response, that also is essential for viral clearance, may offer some cross-protection against other serotypes [68].

HAdV causes a variety of clinical manifestations. In the immunocompetent child, the virus is frequently associated with acute febrile illness, RTI, pharyngitis and gastroenteritis. Neonates and young children may also develop pneumonia and extrapulmonary involvement is sometimes seen. HAdV mainly causes RTI in adolescence but certain serotypes have been associated with more severe respiratory disease and pneumonia [66, 69]. In immuno-compromised individuals, the infections comprise many different clinical manifestations and may sometimes prove fatal due to respiratory failure or disseminated disease [70, 71].

HAdV infections occur worldwide and do not exhibit any clear seasonal pattern but increased activity is sometimes seen during the summer months.

1.2 MEASLES

virology, epidemiology and clinical presentation

1.2.1 Virology

Morbilli virus is the causative pathogen for the highly contagious infection known as measles. This enveloped negative sense non-segmented RNA virus is a member of the *Paramyxoviridae* family and the genome codes for six proteins of which two glycoproteins on the viral surface are key players for host cell interactions. Firstly, the hemagglutinin protein is essential for the binding to a variety of host cells such as lymphocytes, monocytes, dendritic cells and epithelial cells, and as consequence the virus can infect a large variety of cell lines and organs. This glycoprotein is also the target for the specific neutralizing antibodies that are developed after primary infection hence establishing life-long immunity by preventing viral docking to host cell receptors. Secondly, the surface fusion-protein enables entry into the host cells [72, 73].

1.2.2 Historical aspects

Historical evidence suggests that morbilli virus originated from rinderpest virus (RPV), a viral disease of cattle that was declared eradicated by WHO in 2011 as a result of a successful vaccination program. The domestication of cattle, dating several thousand years back in time, probably led to the appearance of a zoonotic version of RPV that through time evolved into the morbilli virus we know today. It is estimated that, due to the life-long immunity after primary infection, a critical mass of 250.000-500.000 densely populated individuals are needed in order to sustain the human-to-human transmission chain of measles [74]. This precondition, together with the fact that measles lack natural animal reservoirs, meant that the prerequisites for the epidemic spread of measles came along with the birth of the modern civilization. The virus has presumably been circulating sporadically within human settlements for several thousands of years, but it remained unnoticed in historical records until the 10th century when Rhazes of Baghdad described the different clinical features of small pox and measles [75, 76]. In the past millennium, the epidemiology of measles has been strongly interconnected with the urbanization and migration of an ever-growing human population. The devastating consequences of this interaction are well documented through the introduction of the virus into the New world leading to a significant decimation of the native populations. From this time historical records also provide more detailed characterizations of the clinical course of measles, for example portrayed by the physician Thomas Sydenham in 1670. During a measles outbreak in Boston he wrote:

"we are to observe, that at this Time the Fever, and Difficulty of Breathing are increased; and the Cough grown so cruelly troublesome, as to hinder Sleep Day and Night"

1.2.3 Epidemiology and transmission

Measles is highly contagious and a single case may transmit the infection to an average of 9-18 individuals in an immunologically naïve population thereby topping the list of communicable diseases in terms of contagiousness. Before the introduction of the measles vaccine in the 1960s, it is estimated that around 90% of the children were infected before the age of 15 [77].

The 2-dose regime of measles vaccine has greatly reduced the burden of disease. WHO has estimated that since the year 2000, approximately 21 million lives have been saved through measles immunisation (primarily children under the age of 5). Measles accounted for around 2 million deaths globally every year in the pre-vaccination era. Successful vaccination campaigns have brought down the numbers to around 100 000 deaths annually. Unfortunately, data from 2018 points towards an increase again, with an estimated 140 000 measles related deaths. Previously, the elimination of endemic transmission was reported from some countries, but new extended outbreaks across regions have led to the resurgence of endemic measles again [78, 79].

The main route of transmission is through small aerosolized respiratory droplets that may remain suspended in air for up to two hours in a confined space. Thus, transmission to naïve individuals may occur, in for example waiting rooms, up to two hours after an infective individual visited the premises [80, 81]. In the pre-vaccination era, the epidemiological pattern of measles was constrained to up to 5 yearlong cycles with peaks occurring during wintertime and spring each year, in a temperate climate. Environmental factors such as ambient temperature may have contributed to the seasonal variation but also the successive depletion of susceptible individuals following continuous outbreaks. Few studies have evaluated the impact of meteorological factors on the incidence of measles since the introduction of the vaccine. Precipitation, RH and outdoor temperature (both high and low) may have an impact on the activity of measles according to some reports but the findings seem to be dependent on the geographical location. However, today the seasonal pattern is often more irregular than in the pre-vaccination era [82-85].

1.2.4 Clinical presentation and complications

The incubation period of measles is normally estimated to be around 10-13 days. A systemic review by Lessler et al. found the median incubation period

to be 12.5 days but it has been reported to be up to 23 days [86, 87]. The clinical course is well described and summarized in Figure 3. Primary measles infection in immunologically naïve individuals (i.e. naïve infection) is characterized by the onset of high fever, usually accompanied with either cough, coryza and/or conjunctivitis within a day or two. The typical macopapular rash starts at day 3-4 after the onset of fever, at which time the pathognomonic Koplik´s spots might be detected on the buccal mucosa. Symptoms usually resolve within a week after rash onset in uncomplicated cases.

Severe complications following the naïve infection served as an important motivator for the introduction of the measles vaccine. Frequently observed complications to naïve infection are secondary bacterial infections such as pneumonia and acute otitis media. Neurological complications are the most feared and include acute disseminated encephalomyelitis (ADEM) which develops a few weeks after the infection and measles inclusion antibody encephalitis (MIBE), a progressive and fatal complication recognized in immunosuppressed individuals within a few months of the primary infection. Subacute sclerosing panencephalitis (SSPE) is a devastating complication affecting roughly 1:100 000 cases with a higher risk in those who experience naïve infection in early childhood. SSPE is caused by the production of mutated virions and usually strikes several years after the naïve infection. A recent study from California, reviewing cases of SSPE over a period of 17 years, calculated an alarmingly high incidence (1:1367 cases) in unvaccinated children under the age of 5 [88]. This illustrates the importance of continuous vaccination against measles in early childhood to prevent naïve infections during infancy.

1.2.5 Diagnosis

Before the vaccination era, measles was a common disease. The diagnose was based on clinical evaluation alone and included the presence of the typical measles rash, high fever and either cough, conjunctivitis or coryza. Today, in a high vaccination coverage setting, this clinical triad has unsatisfactorily low sensitivity, as these clinical symptoms most likely originate from other viral illnesses. Laboratory methods are therefore required in order to diagnose a patient with measles. Previously, serology with detection of measles-specific IgM- and lack of IgG antibodies in acute sera, were used for the diagnosis. Nowadays, qPCR is used to detect measles virus RNA in either nasopharynx, urine and/or serum samples.

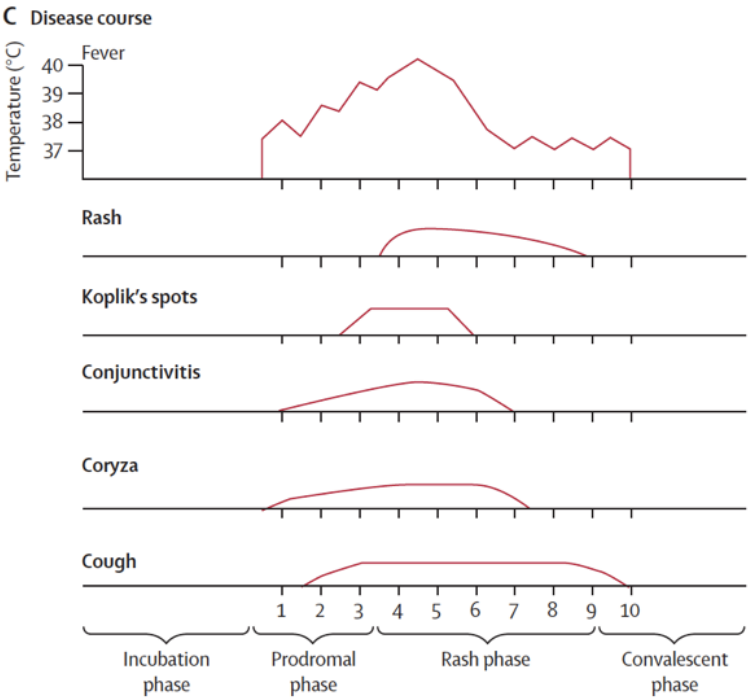

Figure 3. The clinical course of measles in an immunologically naïve individual. Moss W.J. Lancet. 2017 [84]. Copyright. Reprinted with permission from Elsevier.

1.2.6 Breakthrough infections

Since the introduction of the vaccine there has been an increasing number of reports of measles in previously immunised individuals. The term primary vaccine failure has commonly been used for individuals who failed to seroconvert after vaccination. Secondary vaccine failure (sometimes also referred to as modified measles in the literature) has been used to describe measles in previously immunised, and is plausibly explained by waning immunity or vaccination long ago [89]. The terminology within this field has been unclear and furthermore, the term modified measles may be confused with vaccine-modified measles, a term used for the natural infection, with mild clinical symptoms, that occurs after immunisation with the vaccine strain. Thus, in this thesis we will use the term *breakthrough infection* to define measles in patients with previous immunisation. Measles in immunologically

naïve individuals will be defined as having *naïve infection* (primary infection). In the last decade there have been reports of breakthrough infections in health care workers, usually after exposure to infectious measles patients [90-92]. Some studies have also demonstrated that a majority of measles cases during an outbreak in an area with a high vaccination coverage are expected to be breakthrough infections [93, 94]. The clinical presentation of breakthrough infections is not well characterized and although onward transmission from these cases seems to be rare the available data is limited and more studies are needed.

1.3 BACTERIAL PATHOGENS

1.3.1 Streptococcus pneumoniae

It is more than 100 years ago since *S. pneumoniae* was first isolated and declared a cause of pneumonia. To date more than 100 serotypes have been identified of which some are associated with invasive disease and targeted in pneumococcal vaccines. Despite the access to effective antibiotics and modern conjugate vaccines, *S. pneumoniae* prevails as a major cause of morbidity and mortality worldwide [2]. It is still considered to be the leading pathogen in CAP but the detection rates in blood, sputum and nasopharyngeal cultures remain unsatisfactorily low in symptomatic patients. *S. pneumoniae* is also involved in other clinical infections such as otitis and meningitis. Pneumococci may asymptomatically colonize the upper respiratory tract, especially in children. In the adolescence, colonization seems to be less common but the frequency varies between different reports. Compared to cultures, molecular methods have yielded higher detection rates of pneumococci in nasopharyngeal samples in both symptomatic and asymptomatic individuals. The clinical relevance of a positive detection by the latter method is up for debate [95-99].

1.3.2 Haemophilus influenzae

This gram-negative bacterium is commonly associated with pneumonia in the elderly but also in patients with chronic lung conditions such as chronic obstructive pulmonary disease (COPD). Other clinical conditions such as meningitis and epiglottitis are fortunately infrequent nowadays due to the childhood vaccine against the encapsulated *H. influenzae type B*. *H. influenzae* and *Mycoplasma pneumoniae* are next to *S. pneumoniae* the most frequently detected bacterial pathogens in adults with CAP. Carriage of *H. Influenzae* in the upper airways is rare in healthy adults but depending on sampling location

and methods used for identification, rates may range from 1-30% [26, 100, 101].

1.3.3 Mycoplasma pneumoniae and Chlamydophila pneumoniae

Mycoplasma is a common cause of CAP in young adults and is caused by a bacterium without cell-wall hence not susceptible to beta-lactam antibiotics. It is the most common cause of atypical pneumonia. Mycoplasma commonly causes mild RTI but may progress to pneumonia with high fever, bilateral infiltrate and hypoxemia which may require hospitalization. An overactive immune response probably contributes to more severe clinical manifestations. The incubation period varies from one to three weeks. Mycoplasma infections occur worldwide across all seasons and smaller peaks are occasionally seen in the autumn, sometimes coinciding with social gatherings such as school start.

Chlamydophila pneumoniae is also considered to be a pathogen causing atypical pneumonia. It is an obligate intra-cellular bacterium and may cause both mild RTI and pneumonia. The typical clinical course however is a prolonged period of URTI that usually is self-limiting.

1.4 VIRAL TRANSMISSION

The abundance of different respiratory pathogens with varying clinical manifestations entails that the routes and mechanisms of transmission are complex and multifactorial. Environmental factors, weather conditions, social behaviour, infectious dose and host factors (susceptibility as well as local and systemic immunity) may all influence the likelihood of contracting or transmitting a respiratory virus. Three basic and generally accepted routes of transmission are: contact, droplet and aerosol transmission.

1.4.1 Contact transmission

In this case viruses are deposited from an infectious person to a susceptible individual through direct contact, usually via the hands or other contaminated parts of the body. A basic principle for transmission is the inoculation of virus through the mucosa (of the airways or the conjunctiva). Thus, the pathogen can be transferred through direct contact or through self-inoculation. The indirect route of contact transmission is also common and refers to virus being transferred through an intermediate such as fomites in the surroundings. Toys and fomites in the day care environment are often contaminated by various

pathogens which is demonstrated in a recent Danish study that detected a variety of respiratory viruses by PCR in this setting, especially on toys and pillows [102].

1.4.2 Transmission through droplets

Droplet transmission are caused by the formation of droplets expelled from the respiratory tract during respiratory activities (i.e. coughing and sneezing). These particles, produced from saliva, mucus and expectorate, are above 5 μm in size (diameter), a cut-off that is generally accepted and that distinguishes droplets from smaller aerosol particles. This size definition is also adopted in the WHO guidelines regarding infection control [103]. Droplets differ in size but may measure up to 500 μm when produced from an infectious individual [104]. The droplets cannot remain suspended in air due to the mass and the force of gravity hence transmission through air is limited to the close proximity (usually about 1 meter). As a consequence, virus-containing droplets are deposited on fomites in the close surroundings or may settle on a nearby individual. Naturally, the number of produced particles from respiratory activities are dependent both clinical symptoms as well as host- and environmental factors. As early as in the 1940s, Duguid et al. found that a sneeze produced up to one million droplets, one cough around 250 000 droplets but that only 250 droplets were expelled during a one minute talk [105]. However, estimations of the number of droplets or microorganisms that are released during different activities have been lower in some reports (Table 2) [106]. Although there are discrepancies in the literature, it still reflects the impressive quantity of infectious droplets that can be expelled by a person with ingoing respiratory tract symptoms.

Table 2. *An approximation of the number of droplets or airborne microorganism that are expelled during certain activities [106].*

Activity	Number of particles produced
Per sneeze	40 000
Per bowel evacuation	20 000
Per vomit	1000
Per cough	710
Per 100 words	36

1.4.3 Aerosol transmission

Aerosol transmission refers to expelled infectious droplets with a diameter <5 μm. Due to the small mass and size they may remain airborne for an extended period of time allowing long range transmission. A few considerations are essential regarding aerosol transmission. Firstly, the size of the exhaled or expelled particle is crucial for the time it can be suspended in air. Secondly, when expelled, the degree of desiccation of the aerosol particle is essential. Thus, environmental factors such as ambient humidity may transform the mass of the particle through evaporation (or humidification) hence prolonging (or shortening) the airborne phase. Thirdly, when inhaled, the diameter of the aerosol particle influences where it will be deposited in the respiratory tract.

Calculations using Stoke´s law show that the settling velocity of aerosols in still air increases with size and accordingly, a 3 meter fall in still air would take 4 minutes for a 20 μm particle but 65 minutes for a 5 μm particle [107]. Despite these calculations, the transmission and infectivity of aerosolized particles from the human airways are dependent on more factors such as; the amount of water and moisture within the aerosol particle, the number of infectious particles it contains, the severity of the respiratory symptoms and the rate of desiccation. Furthermore, still air is practically a non-existing state in most settings where humans reside which means that the particles will be exposed to the forces of any moving air mass.

A few other facts regarding aerosol transmission are also of interest. The size of the aerosolized particle is not only important for the settling velocity. It also affects where it is deposited in the airways of a susceptible individual. Particles with a size of >10 μm are usually stuck in the thick mucus lining the upper airways while particles <10 μm may penetrate deeper into the airways. Particles with a size <2 μm can easily reach the alveolar region. It is also noteworthy that, in less than a second a large droplet of 50 μm may fully desiccate why even larger particles may rapidly diminish to a diameter below 10 μm (sometimes referred to as droplet nuclei). This would allow it to remain airborne or, if already settled, become airborne again. In addition to this, a study by Cole et al. showed that even large droplets of 100 μm in diameter might remain airborne for a prolonged period of time if the force of an upwards moving airmass in a room is strong enough [106, 108-110].

1.5 METEOROLOGICAL FACTORS AND VIRAL TRANSMISSION

The seasonal patterns of respiratory viral infections became apparent to mankind already in an early stage of history. Even long before the identification of any causative pathogens, it was obvious that cold weather and close social interactions and crowding somehow enigmatically seemed to be interconnected with contagious diseases. Numerous epidemiological and experimental studies have to date partly elucidated some of the important factors behind the seasonality of respiratory viral infections. Nevertheless, there is still no single variable that satisfactorily explain this interesting phenomenon.

Several hypotheses behind the seasonal outbreaks of influenza and other respiratory viruses have been put forward. Some of these are accepted as important contributing factors such as antigenic drift and shift of influenza virus, susceptibility and immune status within the population as well as social behaviour. Meteorological parameters have also been investigated and to what extent they may contribute to the epidemiological patterns seen. In the temperate climate zone, the annual peaks of influenza coincide with the cold winter months whereas humid and rainy conditions are associated with an increased influenza activity in the tropics [16].

1.5.1 Outdoor temperature

Outdoor temperature (degree Celsius) is a fairly easy physical property to comprehend but nevertheless infinitely complex by being involved in most of the biological, physical and chemical processes occurring on this planet. In Sweden, as in other countries situated in the Northern hemisphere, outbreaks of some of the respiratory viral pathogens, and especially influenza, are constrained to the period with low temperatures. Low temperature has been associated with increased incidence and transmission of influenza in both epidemiological, experimental and animal studies. A relationship between low temperature and increased influenza incidence has been presented in several epidemiological studies [111, 112]. Animal models, showing an increased rate of influenza transmission at low temperature (and the opposite during high temperature) as well as experimental studies describing a prolonged viability and survival of influenza virus at low temperature, has supported epidemiological observations [113-115]. Furthermore, studies have also suggested an association between inhalation of cold air and the weakening of the local defence of the nasal mucosa [116]. Altogether, it is clear that cold

weather is related to increased influenza activity, at least in the temperate zone, and possibly also the activity of other viruses causing RTI. Nevertheless, epidemics also occur in the tropics where the outdoor temperature is markedly higher and sometimes without seasonal fluctuations, suggesting that cold air alone cannot fully explain the seasonality. Perhaps the interaction between outdoor temperature and other meteorological factors are of more interest. Accordingly, the research within this field has focused on the role of humidity in the past decade.

1.5.2 Humidity

Humidity (referred to as outdoor humidity here if not stated otherwise) is the concentration of water vapor (the gaseous state of water) in the air. The physical laws of humidity are complex and somewhat out of the scope for this thesis. However, for description purposes, three different types of measurements of humidity are important to grasp: **absolute humidity (AH)**; which describes the total mass of water vapor within a given volume/unit of air irrespective of temperature (g/m^3); **relative humidity (RH)**; a ratio describing the water content in a gas, relative to the maximum capacity of the water vapor that a gas can hold, at a given temperature (%); and **specific humidity (SH);** a mixing ratio describing the mass of water vapor to the total mass of a specific unit of air. AH can be expressed in different meteorological measures such as vapor pressure or mixing ratio/specific humidity and in this thesis vapor pressure is used as a measurement of AH.

The role of humidity in shaping seasonal outbreaks of RTI has been increasingly studied. Earlier reports indicated that low RH is favourable for the spread of influenza virus and also promotes survival of the virus outside the human body [114, 117-119]. Low RH was therefore suggested to contribute to increased transmission during cold weather through evaporation of aerosol particles favouring long range transmission. However, RH is a temperature dependent ratio and does not always satisfactorily reflect the actual amount of water vapor within a given volume of air. Also, RH may sometimes be higher during the winter compared to the summer in the Northern hemisphere. Accordingly, when assessing the relationship between climate factors and the transmission of respiratory viral pathogens, the use of RH as the only measurement of humidity is ambiguous. More recent studies have instead focused more on AH, that irrespectively of temperature, may provide a more accurate measurement of humidity at any given point. The correlation between low AH and increased influenza activity is also stronger compared to RH, according to some reports [120, 121]. To clarify the relationship between temperature, RH and AH, an example is given in Table 3. Note that even tough RH is higher during cold weather, the total amount of water vapor is much

lower (in terms of AH) compared to when the temperature is higher, meaning that the air is significantly drier.

Table 3. *Weekly averages of temperature, relative humidity and absolute humidity (vapor pressure) during three different seasonal time points at the local weather station in Gothenburg.*

Outdoor temperature (°C)	Relative humidity (%)	Absolut humidity (hPa)
0	88	5
10	73	9
20	67	16

1.5.3 Precipitation and wind

Rainfall (mm) and wind speed (m/s) are meteorological parameters that might be, depending on where you reside geographically, much appreciated or a source of endless complaint. Regarding their role in the seasonality of RTI, the literature is more scarce compared to temperature and humidity. An association between rainfall and RSV and influenza has been reported in several studies but with conflicting results with both positive and negative correlations being presented [16, 122]. The discrepancies are probably due to different geographical study sights (tropical vs subtropical regions). The impact of precipitation on the seasonality of RTI in the Northern hemisphere is poorly investigated but the irregular pattern of rain, occurring during all seasons, makes any correlation difficult to assess. The effect of wind speed on the transmission respiratory viruses is also difficult to estimate. Strong winds will likely mean that pathogens may remain airborne but on the other hand disperse rapidly thereby decreasing any risk of long-range transmission. The swift fluctuations of wind speed on a daily basis also make correlations with the incidence of RTI troublesome. A study in Greece reported a significant correlation between RTI and the chilling effect of wind whereas other studies have found no relationship [123, 124].

1.6 ASPECTS ON TRANSMISSION OF RESPIRATORY VIRUSES

The routes of transmission may vary to some extent between different respiratory viruses. Of the pathogens discussed in this thesis, measles and influenza are considered to be the most contagious, which is facilitated by airborne transmission. This is also reflected in the basic reproduction number (R_0), that basically describes how many secondary cases that are produced from one infected individual within a susceptible population. Consequently, the R_0 is affected by the contagiousness of an infected individual, the infectivity of the virus and the susceptibility within the population. As previously mentioned, measles is the most contagious agent we know of with a R_0 of 12-18 (in an immunologically naïve population) whereas influenza has an estimated R_0 of 2-3. Both these pathogens have, in observational and experimental studies, been shown to spread through aerosolized particles with a diameter <5 μm, hence allowing long range transmission [125, 126]. However, both viruses may be transmitted through direct contact or droplets as well. The formation of small aerosolized particles suggests that the transmissibility could be affected by environmental factors such as outdoor weather conditions.

Other respiratory viruses such as HRV, RSV, PIV, HMPV, HCoV and HAdV are generally considered to be transmitted through droplets or close contact [127]. However, some reports indicate that the formation of aerosolized particles could be implicated also in the spread of HRV, RSV, HAdV as well as MERS-CoV and SARS [128-132]. If airborne transmission occurs in the transmission chain of COVID-19 is still unknown. Nevertheless, the impact of environmental factors on transmission cannot be excluded for many of these viruses. Also, the transmission routes are likely to overlap at many times. The classification of respiratory viruses as being strictly transferred through a specific route could therefore be hazardous in terms of risk assessment, especially in the light of the continuous genetic evolvement of viral pathogens.

1.7 POLYMERASE CHAIN REACTION

Polymerase chain reaction (PCR) is nowadays commonly used in laboratories all over the world. It has revolutionized the possibilities for microbial detection and plays a crucial part in modern clinical diagnostics. The method, developed by Kary Mullis in the early 1980s, is basically an amplification of DNA into millions of copies allowing numerous subsequent analysis to be done such as pathogen identification in various clinical specimens. The PCR process can be summarized in the following steps:

1/ heat denaturation of double-stranded DNA leading to single DNA strands

2/ alignment of primers to single DNA strands

3/ the extension of the primer by DNA polymerase resulting in two copies of the original DNA

The process is than continued with a new cycle of denaturation, annealing and elongation, usually about 40 times, amplifying large amounts of the targeted DNA. The basic steps of the process are displayed in Figure 4.

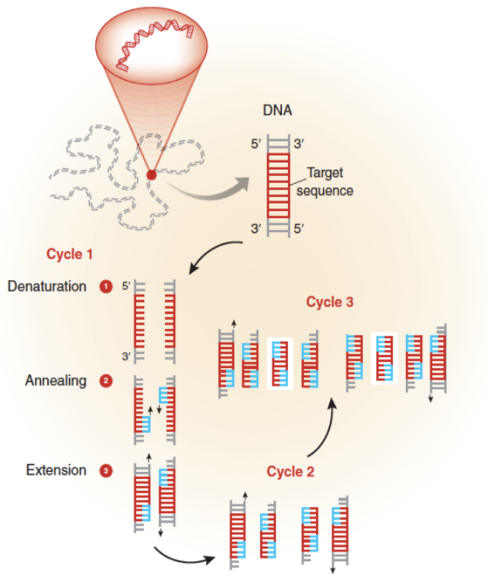

Figure 4. The basic steps of PCR. Garibyan et al. J Invest Dermatol. 2014. Reprinted with permission from Elsevier.

Nowadays qPCR is used extensively in microbiology diagnostics. There are some important advantages with qPCR compared to the original PCR method that merely allowed the detection of the targeted DNA sequence. Firstly, it is possible in real-time to follow the synthetization of the targeted DNA product through fluorescent dyes or DNA probes that illuminates in correspondence to the amount of copies produced during the process. Secondly, the produced light signal can be plotted as a curve allowing a quantification of the amplified target DNA, as measured by the number of PCR cycles (cycle threshold value). This will allow a semi-quantitative estimation of the original amount of viral DNA that were present in the biological sample. By using different fluorescent dyes, amplification of multiple gene targets (multiplex PCR) can be performed in the same run generating different curves for interpretation.

Quantitative PCR offers a high sensitivity and the technique is suitable for many biological specimens. The quantification of the pathogen load is essential in clinical diagnostics both when estimated by the generated cycle threshold value (Ct-value) or when the original amount of the targeted gene is plotted and compared to control samples that contain a known pathogen load (allowing a more exact estimation of the pathogen load in the patient sample). Also, bacterial detection by PCR is not hampered by antibiotic treatment if initiated before sampling.

A few limitations are worth mentioning. The highly sensitive technique means that any contamination of the biological sample may create a disturbance in the PCR process sometimes leading to false or misleading results. Moreover, it is exclusively the targeted gene that can be detected and deviation from the investigated nucleotide sequence may lead to a false negative result. The primers might also attach to a sequence similar to the targeted gene, leading to a false positive result [133-135].

The detection of respiratory viruses in airway samples by qPCR has become an important diagnostic tool for clinicians. However, the results generated by this sensitive technique can be difficult to interpret. A positive detection for a respiratory virus is likely relevant in patients with ongoing symptoms of RTI. However, a detection may also represent prolonged shedding of virus after a previous symptomatic infection or an ongoing asymptomatic infection. It is therefore essential to always evaluate the clinical relevance of a positive finding. Especially since previous studies of the detection rate of respiratory viruses in asymptomatic children exceeds 30% in some reports [136, 137]. The prevalence in asymptomatic adults seems to be lower but the number of studies in this field are still limited [138, 139].

2 AIMS

Overall aim

To explore and describe the epidemiology, clinical presentation and transmission-pattern of respiratory viral infections by utilizing PCR-based laboratory methods.

Specific aims

- to investigate the relationship between outdoor weather conditions and the incidence, seasonal variation and the onset of the annual outbreak of influenza and other respiratory viruses, in our geographical region with a temperate climate (**paper I**)

- to study the detection rate of respiratory viruses in nasopharyngeal samples by PCR in healthy adults without symptoms of respiratory tract infection, in order to interpret the clinical relevance of a positive detection in both asymptomatic as well as symptomatic adults (**paper II**)

- to describe the clinical presentation and laboratory findings in patients with naïve measles infection and breakthrough infection, respectively, and to investigate the transmission chains of the investigated outbreak (**paper III**)

- to study the etiology of lower respiratory tract infections in adult hospitalized patients, with focus on clinical presentation and outcome of viral infections (**paper IV**)

3 PATIENTS AND METHODS

3.1 PATIENTS AND STUDY DESIGN

The study populations in this thesis mainly included adult subjects. However, paper I comprises all clinical samples that were referred to the Department of Clinical Virology, during the study period. Hence, even specimens from children were included in this paper. Children also participated in the study of the measles outbreak in paper III. A unique study population was used for each of the studies. An overview of the study participants is depicted in Figure 5.

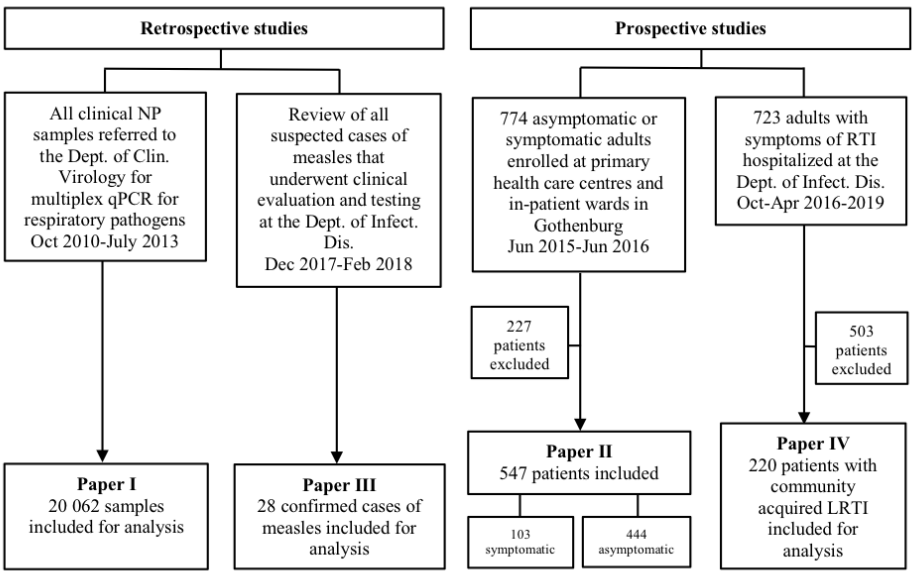

Figure 5. An overview of the study participants in this thesis.

3.1.1 Paper I

This *retrospective observational study* included all clinical nasopharyngeal swab samples, referred to the Department of Clinical Virology at Sahlgrenska University Hospital between October 2010 and July 2013, for the detection of respiratory pathogens using a multiplex real-time PCR panel. The samples covered all age groups. No medical history or clinical data were extracted or accessed during collection and analysis of data. All referred samples during the study period were included in the study (hospital inpatients, primary health care centres and hospital outpatient clinics) and there were no specific criteria for inclusion or exclusion. Meteorological data were obtained from the Swedish Meteorological and Hydrological Institute (SMHI). The data series originated from the local weather station in Gothenburg (situated 5 meters above sea level, at Latitude: 57.7157N, Longitude: 11.9925E) and included daily averages of: outdoor temperature (degree Celsius), vapor pressure (hPa), relative humidity (%), wind speed (meter/second) and precipitation (mm). These parameters were then recalculated into weekly averages.

3.1.2 Paper II

In this *prospective observational study*, nasopharyngeal swab samples were collected, by a study nurse, from adults with and without ongoing symptoms of respiratory tract infection, during 12 consecutive months (June 2015 to June 2016). Study subjects were recruited from three different primary health care centres in the Gothenburg area and two hospital in-patient wards at Sahlgrenska University Hospital.

Inclusion criteria for *asymptomatic* study subjects were: ≥18 years of age and absence of symptoms consistent with respiratory tract infection two weeks prior to enrolment. Exclusion criteria were: inability to provide an accurate history, development of symptoms of RTI within 4 days of sampling, fever, diarrhoea or antibiotic treatment two weeks prior to sampling, and individuals residing in a healthcare facility (i.e. nursing home or residential home). *Symptomatic* study subjects formed a reference population. Inclusion criteria were: age ≥18 years and symptoms of RTI (see Methods) with a duration of ≤10 days. Exclusion criteria were: inability to provide an accurate medical history, admission to hospital in the preceding ten days and admission from a health care facility. A standardized study-specific questionnaire including clinical and laboratory data as well as a symptom score was recorded at time of sampling and by telephone at follow-up (FU) day 7 in a web-based case report form, which constituted the study database. All participants provided written and informed consent.

3.1.3 Paper III

In this *retrospective observational study*, demographic and clinical data on all laboratory-confirmed cases of measles were obtained from medical records. All confirmed cases (older than 1 year) were also offered a follow-up visit (FU) 4-8 weeks post infection for serum sampling and re-analysis of specific measles antibodies. Based on clinical and laboratory data combined with history of previous immunisation, the cases were categorized into three groups: naïve infection, breakthrough infection or vaccine infection.

3.1.4 Paper IV

This *prospective observational cohort study* included adult patients hospitalized with LRTI and was conducted at the Department of Infectious Diseases at Sahlgrenska University Hospital. The recruitment period was from October to April during three consecutive winter seasons (2016-2019). A study nurse collected a nasopharyngeal swab sample (if not already done by clinician) for the detection of respiratory pathogens by multiplex real-time PCR. Inclusion criteria were: ≥18 years of age, symptoms of LRTI (see Methods) with a duration of symptoms of ≤10 days. Exclusion criteria were: inability to provide an accurate medical history, hospital admission in the past ten days, or admission from a health care facility. Patients with a positive viral detection also completed the same standardized study-specific questionnaire used in paper II, after providing a written and informed consent. A retrospective review of medical records was also done in all patients included in the study.

3.2 METHODS

3.2.1 Definitions in the studies of respiratory viral infections

URTI was defined according to the Wisconsin Upper Respiratory Symptom Survey (WURSS) [140]. It includes at least 1 out of 4 symptoms (nasal discharge, nasal obstruction, sneezing or sore throat) AND at least two of the following: sneezing, headache, malaise, chilliness, nasal discharge, nasal obstruction, sore throat, or cough. The definition of *LRTI* was adopted from the Joint Taskforce of the European Respiratory Society (ERS) and European Society for Clinical Microbiology and Infectious Diseases (ESCMID) and defined as an acute illness, usually with cough as the main symptom AND at least one of the following symptoms from the lower respiratory tract: sputum production, dyspnoea, wheeze and/or chest discomfort/pain [141]. The criteria

for *CAP* was adopted from the British Thoracic guidelines and defined as an acute LRTI with radiological evidence of a new pulmonary infiltrate [142]. In paper II, the standardized study-specific questionnaire contained a symptom score at day 0 and at FU day 7. For URTI the WURSS-score was used and for LRTI the community acquired pneumonia-symptom Questionnaire (CAP-Sym) [143, 144]. The latter was also included in paper IV.

3.2.2 Definitions in the study of measles

In Sweden we adhere to The European Union's (EU) case definitions and laboratory criteria for measles. According to the Swedish guidelines the definition of a *suspected case* was a possibly immunologically naïve individual with possible exposure to measles and at least one of the following symptoms: fever, maculopapular rash, conjunctivitis or respiratory symptoms. For contact tracing purposes, a *contact* was defined as an individual who had spent time indoors together with a laboratory-confirmed case of measles in the period of 4 days before to 4 days after the onset of rash [145, 146].

Naïve infection was a confirmed case (according to the EU definition) of measles in a non-immune person with no history of immunisation or measles infection and undetectable levels of measles-specific IgG antibodies in acute sera at or after onset of rash (if taken within 4 days). This was regardless of whether post-exposure prophylaxis (vaccine or immunoglobulin) was given or not.

A *breakthrough infection* was a laboratory confirmed case of measles in an individual with a history of vaccination and/or positive IgG levels (> 399 mIU/mL) in acute sera at or after onset of rash (if taken within 4 days), regardless of whether post-exposure prophylaxis was given or not. In order to confirm a breakthrough infection, we retrospectively analysed the avidity of IgG antibodies in acute sera and at FU. Confirmation of a breakthrough infection was based on the criteria set up by the World Health Organization (WHO) Global Measles and Rubella Laboratory Network (a confirmed case of measles with high-avidity IgG antibodies in acute sera) [147].

In our study, we expanded the definition of *vaccine infection* used by WHO (defined as an individual with rash but without respiratory symptoms and a history of measles vaccination 7–14 days before rash onset) [147], to also include the detection of measles RNA in nasopharyngeal, urine or blood samples and the subsequent confirmation of the vaccine strain by genotyping.

3.2.3 Multiplex real-time PCR for respiratory pathogens

The method of sampling was the same in all studies of respiratory viral pathogens. The swab (FLOQSwabs™ in paper I-II and ESwabs™ in paper III-IV, COPAN Industries Inc) was inserted into the nasopharyngeal cavity and rotated 360° after which it was placed in a container with proper medium. At the Department of Clinical Virology, it was queued for analysis or frozen at -80 °C until analysed. The multiplex in-house qPCR panel, targeted sixteen viruses and four bacteria and has previously been described in detail [3, 148]. The following respiratory pathogens were included: IFA, IFB, RSV, HRV, HEV, HCoV (-NL63, -OC43, -229E and -HKU1), HMPV, HAdV, PIV 1-4 and HBoV; and the bacteria *S. pneumoniae, H. influenzae, C. pneumoniae and M. pneumoniae*. All samples were analysed using the same technique. In short, nucleic acid from 100 µL specimen was extracted into an elution volume of 100 µL and amplified in 25 µL reaction volumes. After a reverse transcription step, 45 cycles of two-step PCR was performed. Each sample was amplified in 8 parallel reactions, each containing primers and probes specific for 2-3 targets. A Ct-value < 40 was considered as a positive result. In cases with a positive signal for both HRV and HEV with a cycle difference of <5 cycles, the result was recorded as HEV/HRV.

3.2.4 Real-time PCR for morbilli RNA

In paper III, all confirmed cases were positive for measles RNA in NP, blood and/or urine sample by qPCR [4]. Suspected cases underwent clinical evaluation and sampling in the isolation care unit at the Department of Infectious Diseases. Samples were immediately referred to the Department of Clinical Virology for acute analysis.

The PCR method is described in detail in paper III. Briefly, nucleic acid from specimens was extracted and eluted after which real-time PCR was performed in 50 µL reaction volume containing primers (measN1F,CGATGACCC-TGACGTTAGCA; measN1R,GCGAAGGTAAGGCCAGATTG) and probe (measN1P,AGGCTGTTAGAGGTTGTCCAGAGTGACCAG), and Super-Script III Platinum One-Step qRT PCR kit with ROX (Invitrogen). After a reverse transcription step for 30 min, followed by 10 min of denaturation, 45 cycles of two-step real-time PCR was performed. The cycle threshold (Ct) value of measles virus RNA was used as a semiquantitative measure of the viral load.

3.2.5 Anti-measles IgM and IgG immunoassays and avidity testing

All suspected cases underwent sampling for measles-specific IgM and IgG antibodies. Sera was tested using the Enzygnost (Siemens Healthcare Diagnostics Products, Eschborn, Germany) anti-measles IgG and IgM enzyme immunoassays on a BEP 2000 ELISA robot (Siemens Healthcare Diagnostics Products). The results of the IgM-test were reported as either positive, equivocal or negative. IgG antibody levels > 399 mIU/mL were considered as positive.

The avidity of IgG antibodies was tested using a commercial test (Euroimmun, Avidity determination of antibodies against measles virus (IgG), Medizinische Labordiagnostika AG, Lübeck, Germany). The relative avidity index (RAI) was calculated in IgG-positive samples (acute and convalescent sera) and RAI < 40% was defined as low avidity, 40–60% as equivocal and >60% as high avidity.

3.2.6 Sequencing and genotyping of morbilli virus

Measles virus genotyping was performed by sequencing of the C-terminal part of the nucleocapsid gene (N-450) at the national reference laboratory at the Public Health Agency of Sweden, Stockholm, and the sequences were deposited by the reference laboratory in the WHO MeaNS database.

For additional distinction, a 400 nt segment of the hypervariable region (HVR) was amplified using primers MorbHVR_F1 (TTCCGCATTTACGACGACG TGA) and MorbHVR_R1 (GTTCCTTGGCCCTAAGTTTTGT). When needed, a second (inner) PCR was performed using primers MorbHVR_F2 (GTGATCATAAATGATGACCAAGGAC) and MorbHVR_R2 (GTCACCT CGGTCGCTTGTG). A cycle sequencing reaction was then performed using the same primers as used in the amplification.

The sequences were aligned with reference sequences from GenBank and phylogenetic analysis was performed using MEGA7 software [149]. A phylogenetic tree was created which is presented in paper III.

3.3 STATISTICS

A summary of the statistical methods in this thesis is depicted in Table 4. In all statistical calculations a significance level of 0.05 was applied (2-sided). A more detailed description of the statistical methods is found in each paper. However, a few particular considerations are worth further discussions.

Firstly, in **paper I**, the use of meteorological factors had to be carefully assessed before being used in any statistics. Several options were at hand. We could choose to use daily, weekly or monthly averages of meteorological factors for the correlation with the incidence of respiratory pathogens. Daily averages would have been possible to use but would likely have produced an unwanted variance from one day to another, hence disturbing the linear regression model. Furthermore, using daily number of positive NP samples would not theoretically have reflected the activity of respiratory pathogens over time. Also, the number of referred and analysed NP samples decreased substantially during off-work hours (i.e. during the weekends) making the use of daily positive samples inappropriate. The use of monthly averages would on the other hand have masked the seasonal fluctuations of both weather conditions and respiratory pathogens. Consequently, the use of weekly averages was used in our study. Another important consideration was the fact that temperature and AH were strongly intercorrelated. As a consequence, these parameters would not fit in the multiple linear regression model and thus AH (vapor pressure) was removed from the model due to multicollinearity. This statistical problem also had to be addressed in the multiple logistic regression model in **paper II**, exemplified by the co-variance of vaccination against influenza and age.

Secondly, using both continuous and categorical variables in the same multivariable logistic regression model are a challenge. Therefore, in **paper IV**, univariate comparisons were performed using Pearson chi-square for categorical variables and Mann-Whitney test for continuous variables before being incorporated into the multivariable regression model. Odds ratios for continuous variables, obtained in the multivariable regression model, were then recalculated in order to be interpretable.

Table 4. *Statistical methods used in each of the papers.*

Statistical method	**Paper I**	**Paper II**	**Paper III**	**Paper IV**
Pearson correlation coefficient (simple linear regression)	X	X		
Pearson chi-square test *or* Fischer´s exact test		X	X	X
Mann-Whitney test		X	X	X
Multiple linear regression	X			
Multivariable logistic regression		X		X
Positive and negative predictive value				X

3.4 ETHICS

In **Paper I,** no clinical or personal data were recorded and ethical approval was not needed. **Paper II-IV** were approved by the regional ethical review board at Gothenburg University. Dnr 912-14 (**paper II+IV**) and Dnr 409-18 (**paper III**).

4 RESULTS WITH DISCUSSION

4.1 RESULTS PAPER I

4.1.1 Seasonal variation of respiratory pathogens

In this retrospective study, a review of 20062 clinical NP samples, referred for multiplex qPCR for the detection of respiratory pathogens across almost three years, was done. Overall, 52% (n=10579) of the samples were positive for a respiratory pathogen and the five most commonly detected agents were: HRV (14%), IFA (8%), RSV (6%), HCoV (4%) and *M. Pneumoniae* (4%). The weekly incidence of respiratory pathogens during the study period were investigated for seasonal variation and the pattern of the six most frequently detected agents across the study period are shown in Figure 6. All the displayed viruses had strong seasonal variation, peaking during the cold winter months, except from HRV that was prevalent across all seasons.

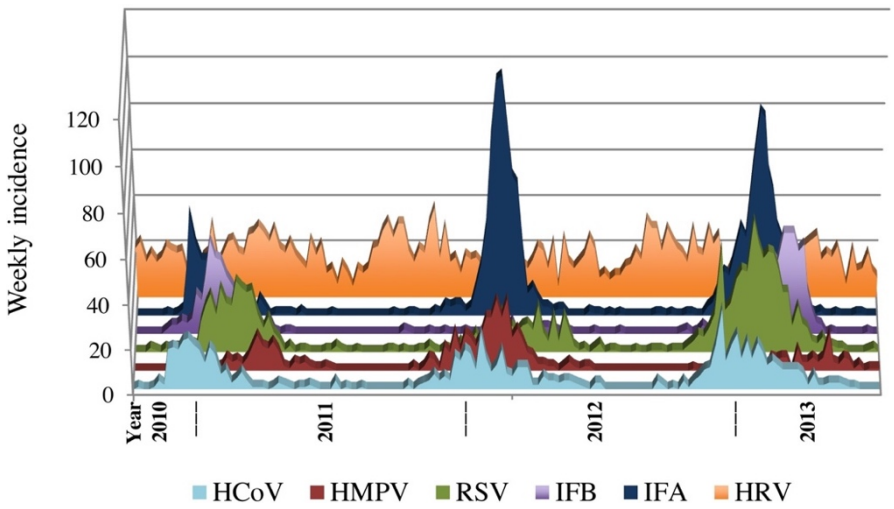

Figure 6. Seasonal pattern of human coronavirus (HCoV), human metapneumovirus (HMPV), respiratory syncytial virus (RSV), influenza B (IFB), influenza A (IFA) and human rhinovirus (HRV), based on the weekly number of positive NP samples by qPCR between 2010-2013. Sundell et al. J Clin Virol 2016. Reprinted with permission from Elsevier.

The seasonal variation of the other respiratory pathogens, included in the multiplex PCR, is displayed in Figure 7. Periods of increased activity of *M. Pneumoniae* were seen, mainly during the autumn. PIV, HAdV and HBoV did not appear to be as strongly wintertime-specific as influenza, with activity stretching from autumn to spring. Any increase of the incidence of these pathogens seemed to be less predictable with infections occurring also during the summer months. HEV displayed a similar pattern as HRV with an all-year activity, albeit with a low overall incidence.

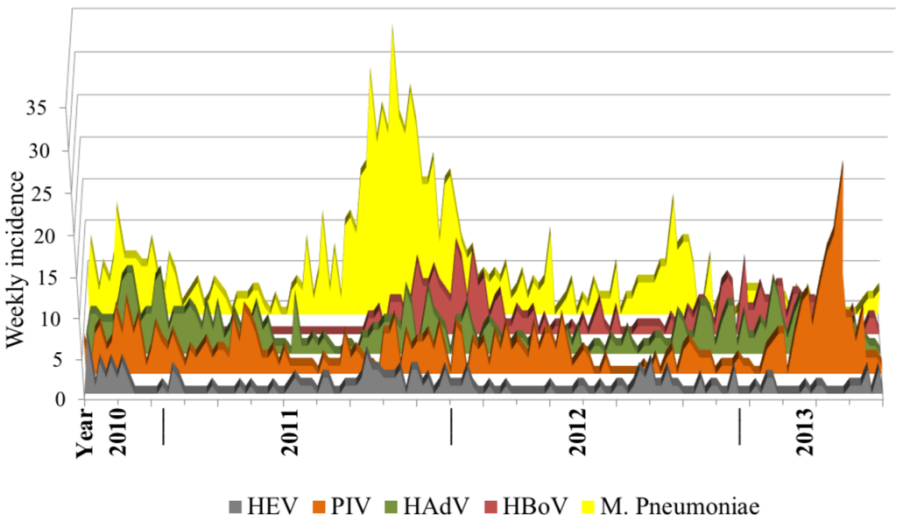

Figure 7. The seasonal pattern of human enterovirus (HEV), parainfluenza virus (PIV), human adenovirus (HAdV), human bocavirus (HBoV) and the bacterium M. pneumoniae, during the study period.

4.1.2 Weather conditions and incidence of respiratory pathogens

In this study we calculated weekly averages of outdoor temperature (°C), relative humidity (%), vapor pressure (hPa), wind speed (m/s) and precipitation (mm). Figure 8 depicts outdoor temperature and humidity in our geographical region, in relation to the weekly number of positive samples of IFA. As noted, there was a strong co-variance between outdoor temperature and AH (vapor pressure), whereas RH in fact was slightly higher during autumn and winter. Wind speed and precipitation are not included in the figure due to the swift variations of these parameters on a weekly basis, without any distinct seasonal behaviour.

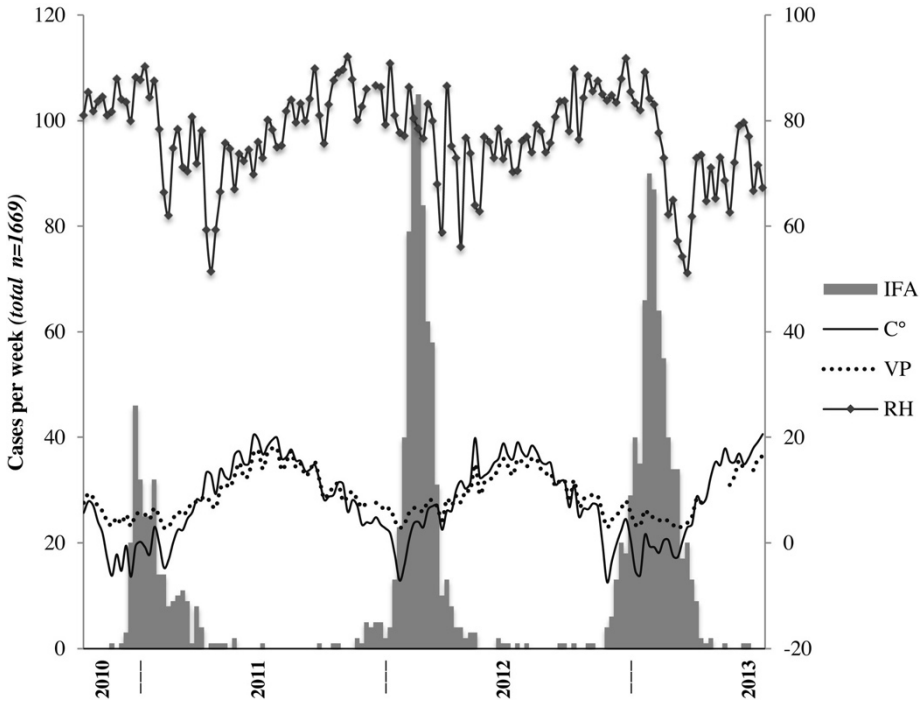

Figure 8. Outdoor temperature (°C), vapor pressure (VP) and relative humidity (RH) in relation to the weekly number of positive samples of influenza A during the study period. Sundell et al. J Clin Virol. 2016. Reprinted with permission from Elsevier.

In univariate comparisons, the weekly incidence of IFA, IFB, HCoV, HMPV, HBoV and HAdV, correlated significantly with both low temperature and low AH. IFA, IFB, HCoV and RSV exhibited the strongest negative correlations. The activity of HRV and HEV were independent of these two weather parameters. A significant correlation between RH and IFB, HCoV, PIV and HEV, were also seen (negatively correlated for IFB and PIV but positively correlated for HCoV and HEV). The meteorological factors that were independently associated with each pathogen in the multiple regression model are presented in paper I. IFA, IFB, RSV, HCoV, HMPV, HAdV and HBoV remained independently associated with low temperature. Not presented in the article, but highlighted in Table 5, is the impact of AH (when temperature was removed from the model) on the weekly incidence of these six respiratory viruses. This also provides an appreciation of the strong intercorrelation of temperature and AH.

Table 5. *Multiple linear regression model with each pathogen as dependent factor and weather conditions (relative humidity, wind speed, precipitation and either temperature* **or** *absolute humidity) as independent factors. Only significant correlations for temperature (***or** *absolute humidity) are presented in each model. The intercorrelation of temperature and AH meant that they had to be tested separately in the multiple regression model (multicollinearity).*

		Significant predictor			**Significant predictor**		
		Temperature (absolute humidity removed)			Absolute humidity (temperature removed)		
Agent	Positive cases	Adjusted R^2	Coefficient	*p*	Adjusted R^2	Coefficient	*p*
IFA	1669	0.22	-1.23	<0.001	0.2	-2.1	<0.001
IFB	812	0.38	-0.86	<0.001	0.33	-1.4	<0.001
RSV	1273	0.34	-0.88	<0.001	0.31	-1.5	<0.001
HCoV	861	0.51	-0.65	<0.001	0.47	-1.1	<0.001
HMPV	584	0.12	-0.2	<0.001	0.12	-0.4	<0.001
HAdV	456	0.28	-0.16	<0.001	0.3	-0.3	<0.001
HBoV	290	0.16	-0.11	<0.001	0.16	-0.2	<0.001

4.1.3 Temperature drop and outbreak onset

During the study period, the onset of the IFA outbreak was preceded by a drop in the average weekly temperature below 0 °C (and vapor pressure below 4 hPa) each season. In order to more thoroughly analyse this relationship, we investigated the impact of the weekly change of meteorological parameters on the incidence of IFA during the outbreak onset each season. The period two weeks prior to the first week with average weekly temperature below 0 °C to the week with maximum IFA incidence, was defined as outbreak each season. We found that the weekly change in average temperature (a temp drop) significantly correlated with the weekly IFA incidence the following week ($p=0.03$). The same association was found for vapor pressure ($p=0.05$).

4.2 DISCUSSION PAPER I

4.2.1 Impact of weather conditions on seasonality and outbreak

The results in paper I showed that the activity of enveloped viruses such as IFA, IFB, RSV and HCoV were confined to the winter months and were strongly associated with low temperature and low AH. Based on our findings, and others, this suggests that the transmission routes of these viruses are not confined to droplet or contact transmission but likely include airborne transmission facilitated by aerosol spread in cold and dry air [128, 130]. Existing data support this mechanism regarding influenza viruses but further studies are needed to explore the degree of airborne transmission in RSV and HCoV infections. Nevertheless, in our study, HCoV had the highest adjusted R squared value which could suggest that airborne transmission of this pathogen cannot be excluded during cold and dry weather. A possible role of this route for HCoV is further supported by previous reports on SARS and MERS-CoV that have proposed that airborne transmission might have occurred, at least in hospital settings [128, 132, 150-152]. Future studies on COVID-19 will likely contribute to an increased understanding of the transmission routes of HCoV in general but also if airborne transmission is implicated in the spread of this novel emerging pathogen.

Increased transmission during winter might not exclusively correspond to the affect that ambient temperature and humidity have on expelled particles through evaporation and desiccation. Also, one could speculate that the lipid layer of enveloped viruses, that has been described to be sensitive to surrounding environmental factors, could have a role in shaping the seasonal variation to some extent [153]. In contrast, the more robust non-enveloped viruses (HRV and HEV) were prevalent all year around in our study and the activity seemed to be independent of temperature or humidity. This suggests transmission routes other than aerosol spread.

Based on our results we can conclude that there is a strong association between cold and dry air and the seasonal activity of IFA in our geographical setting. We found that a weekly average temperature subsiding 0 °C and AH below 4 hPa (VP) preceded the seasonal outbreak onset with approximately one week each season. Based on our retrospective study design, we cannot conclude that these observed trigger points are applicable to other regions with a temperate climate. However, some studies have suggested that a critical level of AH may exist. A US-based study by Barecca et al. found an association between AH below 6 gram of water vapor per kilogram of air (specific humidity) and

increased influenza mortality [120]. A recent study from Oslo in Norway, investigating influenza-like illness as well as positive influenza samples over an 8-year period, reported that the influenza outbreak each season coincided with an average weekly vapor pressure below 4 hPa [154]. No statistical analyses were presented in that study though. Furthermore, a study from Finland also presented that a drop in temperature and AH preceded an increased risk of influenza episodes in a similar geographical setting as ours [112]. It is plausible to believe that a critical level of temperature and AH are likely to differ between geographical regions. Nevertheless, existing data support our findings that a sudden drop in temperature and AH (in our region presumably below approximately 0 °C ad 4 hPa, respectively) may act as an important trigger of the seasonal flue.

4.2.2 The role of humidity

Previous experimental studies have described that low RH favours transmission and viability of influenza virus [114, 118]. This might be true in an experimental setting when climate conditions can be controlled. In our study we can clearly demonstrate that RH is higher during wintertime in our region, which contradicts previous reports stating that low RH favours IFA activity. A recent study from Scotland also found that the activity of RSV and IFA were peaking when RH was high [155]. This could be interpreted as an inverse relationship than that of AH but would still indicate that the air is dry (in terms of AH), irrespective of the calculated RH. Since RH is a temperature-dependent ratio, this parameter may differ substantially between geographical regions and, most importantly, with temperature at any given point. Furthermore, the results in our study demonstrate that AH is more strongly associated with the activity of influenza (and other viruses) than compared to RH, which is in line with a few previous reports [120, 121]. Accordingly, future modelling of the relationship between weather conditions and the seasonality of respiratory viruses should include AH and/or specific humidity, especially since the calculated ratio of RH alone cannot fully explain this phenomenon in an outdoor setting.

4.2.3 Other aspects on transmission

Finally, one could argue that in-door crowding during cold weather is an important driver of viral transmission, facilitated by close human interaction. It is likely that in-door crowding partly could explain increased transmissibility during poor weather conditions. However, a linear relationship between the inactivation of IFA virus and increasing RH has been described [156]. Consequently, if outdoor air during wintertime (with low AH but high RH) are heated indoors, it is assumable that RH would drop significantly as the temperature increases. This would lead to an even drier environment indoors,

with low humidity, that could contribute to continuous viral spread by affecting both droplet size as well as the rate of viral inactivation, despite a comfortable indoor temperature. Furthermore, when considering that even larger particles may desiccate rapidly in dry air and also remain airborne by the force of a moving air mass, possibly by indoor ventilation, one could speculate that there are also prerequisites for effective viral transmission indoors during wintertime [106, 108]. Future studies are needed to further elucidate the complex mechanisms of indoor transmission.

4.3 RESULTS PAPER II

4.3.1 Prevalence of respiratory agents in asymptomatic subjects

The overall aim of paper II was to explore the prevalence of respiratory viral pathogens in nasopharyngeal samples as detected by qPCR, in adults asymptomatic of RTI. Adults with ongoing symptoms of RTI were recruited as a reference population. Of the initially 644 enrolled asymptomatic subjects, a total of 444 were included for final analysis (49 did not fulfil inclusion criteria, 140 were lost to FU and 11 had developed symptoms of RTI at FU). A total of 103 symptomatic subjects were included for analysis, of whom 75 patients (35 with URTI and 40 with LRTI) also completed symptom scoring at FU. The two study-populations were well-matched although chronic lung disease, malignancy and diabetes were more prevalent in the symptomatic group.

The main result was that the detection rate of respiratory viral pathogens was low (4.3%) in the asymptomatic group and that HRV was the predominant finding among these subjects. Pathogens associated with LRTI (IFA, IFB, RSV) were infrequent. No multiple viral detections were registered in either the asymptomatic or the symptomatic group. Findings of *S. pneumoniae* and *H. influenzae* were uncommon. As expected, the overall detection rates of viral and bacterial pathogens were significantly higher in the symptomatic group. The detected pathogens are summarized in Table 6.

4.3.2 Patient factors associated with pathogen detection

To explore if any patient characteristics or demographic features were associated with the likelihood of viral or bacterial detection among asymptomatic subjects, a univariate analysis and a multivariate logistic regression were performed (Table 7). In univariate comparisons, both age ≥65 and vaccination against influenza, were associated with a low probability of viral detection. In the multivariate logistic regression model, age ≥65 was the only factor that remained independently associated with a low probability of viral detection (OR: 0.3; 95% CI:0.1-0.9). Current smoking (OR: 7.3; 95% CI:3.2-17) and presence of any chronic medical condition (OR: 3.6; 95% CI:1.4-9.6) were independently associated with bacterial detection (*S. pneumoniae* and *H. influenzae*). We could not identify any factors that were associated with either viral or bacterial detection among symptomatic subjects.

Table 6. *The frequency of respiratory pathogens detected by multiplex PCR in NP-samples among asymptomatic and symptomatic subjects.*

	Asymptomatic subjects *(n=444)*	**Symptomatic reference subjects** *(n=103)*	***p*-value** [3]
Any pathogen (including bacteria)	49 (11) [1]	51 (50) [1]	<0.0001
Any virus	19 (4.3)	37 (36)	<0.001
> 1 virus [4]	0	0	-
Rhinovirus	14 (3.2)	23 (22)	<0.001
Influenza A	0	2 (1.9)	NC [2]
Influenza B	0	2 (1.9)	NC
Coronavirus	2 (0.5)	6 (5.8)	0.0008
Enterovirus	1 (0.2)	0	NC
Adenovirus	0	0	NC
Parainfluenza virus	0	1 (1.0)	NC
Bocavirus	1 (0.2)	0	NC
Respiratory syncytial virus	0	2 (1.9)	NC
Metapneumovirus	1 (0.2)	1 (1.0)	NC
S. pneumoniae	25 (5.6)	7 (6.8)	0.65
H. influenzae	6 (1.4)	10 (9.7)	0.0001
M. pneumoniae	0	1 (1.0)	NC

[1] *Data presented as n (%)*
[2] *Not calculated due to small numbers*
[3] *Pearson chi-square (or Fischer´s exact test when appropriate).*
[4] *Multiple viral detections*

Table 7. *Multivariate p-values and odds ratios of factors associated with detection of respiratory pathogens among asymptomatic subjects.*

	Univariate	**Multivariate**	
Virus	*p*-value	*p*-value	odds ratio (95% CI)
Age ≥65	0.02	0.04	0.3 (0.1-0.9)
Children at day-care	0.2	0.4	2.2 (0.4-11)
Bacteria			
Current smoking	<0.001	<0.001	7.3 (3.2-17)
Any chronic medical condition	0.05	0.009	3.6 (1.4-9.6)

4.4 DISCUSSION PAPER II

4.4.1 Detection rates of respiratory viral pathogens

Real-time PCR is a sensitive and reliable technique but a positive detection of a respiratory pathogen in NP samples must be evaluated in terms of clinical relevance. Previous studies in asymptomatic children have shown that detection of respiratory viruses is common. Moe et al. found that 30% of children in a day-care setting without symptoms of RTI were positive for a respiratory virus in NP samples [137]. Other studies focusing on asymptomatic children have found detection rates varying from 28% and upwards [157-161]. Our study adds vital information regarding detection rates of respiratory viruses in asymptomatic adults, where available data is limited. We detected a virus in 4.3% of the asymptomatic subjects, a rate which is in line with a report from Self et al. who reported a detection rate of 2.1% in 238 asymptomatic adults compared to 24.5% in a cohort of adults with CAP [139]. Another study also presented a 2% frequency in 50 adults admitted to hospital without symptoms of RTI, however mostly throat samples were collected in that study [162]. A higher rate (7.1%) of respiratory viruses was reported in a study that included 450 asymptomatic adult controls [138]. Varying methods of sampling and the lack of follow up to exclude asymptomatic subjects that developed symptoms post enrolment, may account for the higher rate in that report. In our study, there was no difference in the detection rate of respiratory viruses in asymptomatic individuals sampled in primary health care or in hospital wards (4.3% and 4.1%, respectively). This is in line with a previous Swedish study who reported two viral detections (both HRV) in 100 asymptomatic controls in a similar primary health care setting [163].

When adding our data to a meta-analysis from Jaarti et al., we may conclude that detection of respiratory viruses by PCR in NP-samples from adults without symptoms of RTI is uncommon, with an estimated frequency of less than 5% [164]. This offers new insights into the interpretation of respiratory viral detection by PCR in NP samples. Accordingly, and most importantly, a positive finding in an adult with ongoing symptoms is likely of clinical relevance.

4.4.2 Comments on human rhinovirus

HRV was the most common viral pathogen among the asymptomatic subjects in our study and found in 14/19 (74%) of the positive detections. When evaluating other studies on asymptomatic subjects, including both children and adults, HRV seems to be the predominate agent [137, 139, 158, 162]. This is not surprising when considering that asymptomatic infections are common, re-

infections are frequent due to many circulating serotypes, prolonged viral shedding may occur and infections are seen across all seasons [165, 166]. In fact, when HRV was excluded, the detection rate of other respiratory viruses among the 444 asymptomatic adults was as low as 1%. Based on our findings, and others, we can conclude that respiratory viruses such as HRV and HEV (and sometimes HCoV) are the most frequently detected pathogens in asymptomatic individuals. Other viral pathogens, that are associated with LRTI, are rarely detected in this group. Thus, detection of pathogens such as IFA, IFB, RSV and HMPV are usually associated with ongoing symptoms and of clinical relevance when detected. This is further supported by the findings in paper IV where these viruses were commonly detected in patients hospitalized with viral infections while detection of HRV were infrequent in these group. Also, when adding the data from paper II and paper IV to current literature we may summarize that multiple viral detections, as compared to children, are relatively uncommon in asymptomatic and symptomatic adults. Nevertheless, when dual viral detections are made, HRV, HEV or HCoV are common and may suggestively be of less clinical importance. However, the lack of sequential testing in our studies hampers any strong conclusions.

4.4.3 Factors associated with viral detection in asymptomatic

The results in paper II showed that only age ≥65 remained independently associated with the probability of viral detection. As discussed previously, vaccination against influenza was removed from the multivariate logistic model due to intercorrelation with age (8% of subjects <65 years of age reported vaccination versus 59% of subjects ≥65 years of age). However, vaccination against influenza did not remain independently associated with viral detection (when age was removed from the multivariate model). Few studies have evaluated risk factors associated with the detection of respiratory viral pathogens in asymptomatic adults. Not surprisingly, immunosuppression has been linked to increased detection rates in asymptomatic individuals due to prolonged shedding and impaired viral clearance, especially after hematopoietic stem cell transplantation [167, 168]. Immunosuppression were rare among our study subjects and only 29/444 (7%) reported mild immunosuppression, of which only 1 viral detection was made. A more comparable study to ours found HRV in 2% of asymptomatic adults over the age of 60 supporting our data of a low prevalence in this group [169]. It is assumable that elderly might be less exposed to viral infections compared to younger adults having children at home. A selection bias towards older individuals in our study might also partly explain the results. Also, the limited positive sample size (n=19) precludes any strong conclusions. Further studies are needed within this field.

4.4.4 Aspects on bacterial detection

Asymptomatic nasopharyngeal carriage of *S. pneumoniae* and *H. influenzae* in adults is relatively infrequent although PCR-based methods might yield higher detection rates than conventional cultures [95, 96, 98, 101, 170, 171]. We found *S. pneumoniae* in 5.6% and *H. influenzae* in 1.4% of the asymptomatic adults. When current smokers were excluded, which is a risk factor for pneumococcal carriage, the detection rates dropped to 4.1% and 0.9%, respectively. The overall detection rate of *S. pneumoniae* in our study is lower than that of other reports [95, 101, 170, 171]. Varying sampling techniques may explain some of the discrepancies. Interestingly, the rate of pneumococcal detection was lower in the symptomatic group compared to the asymptomatic. This could plausibly be explained by the fact that almost half of these patients were classified as URTI (few patients with pneumonia) and that the location of sampling (primary health care vs in-patient wards) was disproportional in the two study groups. The detection of *H. influenzae* among asymptomatic subjects was low and comparable to another Swedish study in primary health care [96]. Higher frequencies have been reported however [101]. It is noteworthy that the rate *H. influenzae* was significantly higher among symptomatic patients in our study, suggesting that this pathogen is more commonly detected in patients with ongoing symptoms of RTI. Furthermore, the overall detection rate of *H. influenzae* by PCR in the study cohort of paper IV was significantly higher (15%) when compared to the asymptomatic group in paper II. This may further promote that the detection rate of this pathogen by PCR is more frequent in patients with RTI. Nevertheless, the clinical relevance many times remains elusive.

4.5 RESULTS PAPER III

4.5.1 Outbreak characteristics

This retrospective study describes an outbreak of measles in Gothenburg between December 2017 and February 2018, with special emphasis on clinical and laboratory characteristics in patients with naïve and breakthrough infections. A total of 28 laboratory confirmed cases of measles were diagnosed during the outbreak. The definitions of naïve infection and breakthrough infection used in the study are described in the Method section. Twelve cases were diagnosed as having naïve infection and all fulfilled both clinical and laboratory criteria according to the European union´s case definition. There were sixteen cases of breakthrough infections of whom nine were health care workers. The transmission chain of the outbreak is presented in Figure 9.

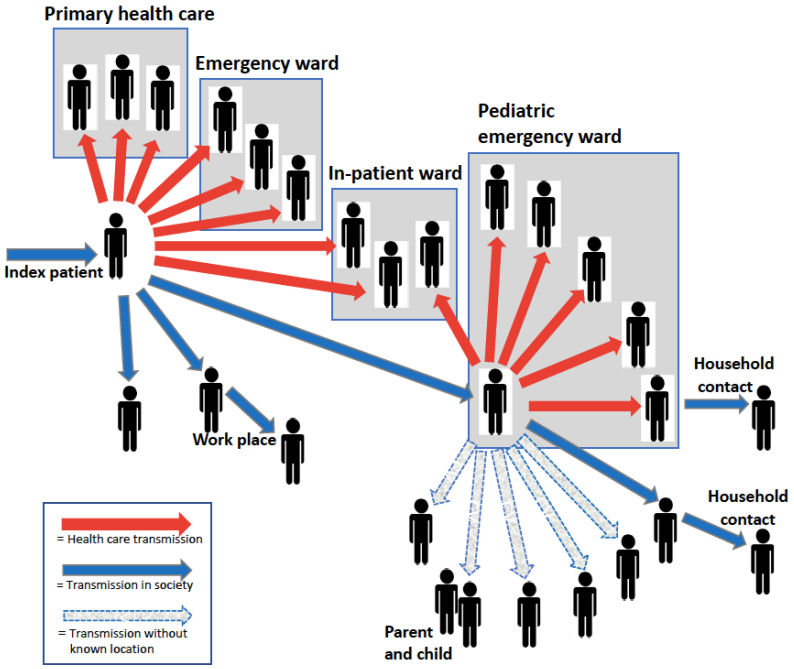

Figure 9. The index case and the following transmission chain of measles during the outbreak in Gothenburg 2017/2018. Graphic layout by Department of Communicable Disease Control, Region Västra Götaland, Sweden.

4.5.2 Naïve and breakthrough infections

Data on naïve and breakthrough infections are summarized in Table 8 and 9. All patients with naïve infections reported no previous immunisation and no history of measles infection. All breakthrough infections reported previous immunisation although documentation was missing in some cases. Detectable levels of IgM antibodies in acute sera were found in 7/12 (58%) of the naïve infections and all were negative for measles IgG except one patient (patient 12 discussed more thoroughly in Table 8 and 9). All breakthrough infections except two) had detectable levels of IgG antibodies (>399 mIU/mL) in acute sera. At FU all of the sampled naïve infections had developed measurable levels of measles IgG whereas patients with breakthrough infections had a boosting effect of the IgG levels following a secondary immune response. A preliminary classification to identify breakthrough infections, based on history of vaccination and detectable measles IgG antibodies (>399 mIU/mL) in acute sera obtained within 4 days after the onset of rash, correctly identified 14/16 (88%) of these infections. Retrospective avidity testing, with the presence of high-avidity IgG antibodies in acute serum samples, confirmed breakthrough infections in cases where material was sufficient for analysis.

4.5.3 Clinical symptoms and viral load

Differences in the clinical presentation of naïve and breakthrough infections are presented in paper III. In summary, patients with naïve infections experienced more pronounced symptoms compared to breakthrough infections. All 28 cases had a macopapular rash. The presence of cough differed significantly between the groups and was found in all patients with naïve infection but only in 4/16 (25%) of the breakthrough infections ($p<0.001$). Analysis of viral load in both NP and urine samples (as estimated by Ct value) showed significantly lower Ct values (higher viral load) in naïve infections compared to breakthrough infections (Figure 10). Two naïve cases with a high viral load in NP samples transmitted measles to 24 individuals (Ct value of 17 and 18, respectively). No onward transmission from breakthrough infections could be identified. In addition to the 28 cases, another 6 patients were diagnosed with vaccine infection during the outbreak. They were previously unimmunised adults and received the first dose of measles vaccine during the outbreak. All experienced relatively pronounced symptoms and 5/6 (83%) were positive for measles RNA in either NP, urine or blood samples, making them difficult to dismiss as a possible naïve infection upon clinical evaluation. However, genotyping later confirmed the vaccine strain. Genotyping was also performed in 25 of the 28 cases with confirmed measles infection showing subtype B3 in all cases. Further sequencing, including the hypervariable region, of 18 of these strains revealed the same B3 strain with no or minimal genetic variation.

Table 8. *Epidemiological data on the 28 confirmed cases of measles during the outbreak in Gothenburg.*

Patient	Infection type[a]	Age group (years)	Doses of measles vaccine received	Fulfilled EU criteria for confirmed case[b]	Infected others
1	N	21–30	0	Yes	Yes
2	N	31–40	0	Yes	Yes
3	N	0–10	0	Yes	Yes
4	N	0–10	0	Yes	No
5	N	31–40	0	Yes	No
6	N	0–10	0	Yes	No
7	N	0–10	0	Yes	Yes
8	N	51–60	0	Yes	Yes
9	N	31–40	0	Yes	No
10	N	0–10	0	Yes	No
11	N	0–10	0	Yes	No
12[c]	N	31–40	0	Yes	No
13	B	21–30	1[d]	No	No
14	B	31–40	2	No	No
15	B	31–40	2	No	No
16	B	31–40	2	No	No
17	B	11–20	1[d]	No	No
18	B	41–50	1[d]	Yes	No
19	B	31–40	2	Yes	No
20	B	51–60	1[d]	Yes	No
21	B	31–40	1[d]	No	No
22	B	21–30	1	No	No
23	B	31–40	1	Yes	No
24	B	31–40	1	Yes	No
25	B	51–60	2	No	No
26	B	31–40	1[d]	No	No
27	B	21–30	1[d]	No	No
28	B	51–60	1	Yes	No

B: breakthrough infection; N: naïve infection; EU: European Union.

a/ Breakthrough infection was defined as a confirmed case of measles in an individual with history of vaccination and/or positive IgG levels (> 399 mIU/mL) in acute serum at or after onset of rash (if taken within 4 days), regardless of whether post-exposure prophylaxis was given or not.
b/ Fulfilled both clinical and laboratory criteria according to the EU case definition.
c/ Patient 12 had no history of vaccination against measles and no history of measles infection. This patient presented low levels of IgG (579 mIU/mL) at first sampling 5 days after onset of rash and had received post-exposure measles vaccine 7 days before onset of rash.
d/ Reported at least one dose of measles vaccine, not documented.

Table 9. *Laboratory characteristics in acute sera and follow-up samples of the 28 confirmed cases of measles during the outbreak in Gothenburg.*

Patient	Infection type[a]	IgM acute sera	IgG (mIU/mL) acute sera	IgG (mIU/mL) FU	Avidity index[b] (%) acute sera	Avidity index[b] (%) FU
1	N	Equivocal	Neg	6,191	NA	54 (E)
2	N	Pos	Neg	10,083	NA[c]	67 (HA)
3	N	Pos	Neg	ND	NA	ND
4	N	Neg	Neg	ND	NA	ND
5	N	Pos	Neg	ND	NA	ND
6	N	Equivocal	Neg	ND	NA	ND
7	N	Pos	Neg	13,454	NA[c]	73 (HA)
8	N	Pos	Neg	ND	NA	ND
9	N	Neg	Neg	3,648	NA	44 (E)
10	N	Pos	Neg	9,420	NA	58 (E)
11	N	Pos	Neg	12,682	NA	ND
12[d]	N	Neg	579	5,204	16 (LA)	45 (E)
13	B	Neg	11,952	29,730	> 99 (HA)	> 99 (HA)
14	B	Equivocal	22,650	> 30,000	94 (HA)	91 (HA)
15	B	Neg	7,040	> 30,000	80 (HA)	89 (HA)
16	B	Neg	508	26,891	65 (HA)	96 (HA)
17	B	Neg	2,513	> 30,000	78 (HA)	92 (HA)
18	B	Equivocal	5,593	ND	ND[e]	ND
19	B	Neg	27,960	> 30,000	99 (HA)	> 99 (HA)
20	B	Equivocal	29,425	28,030	96 (HA)	97 (HA)
21	B	Neg	3,931	> 30,000	85 (HA)	> 99 (HA)
22	B	Neg	4,350	> 30,000	82 (HA)	> 99 (HA)
23	B	Neg	Neg	ND	ND	ND
24	B	Equivocal	28,980	> 30,000	90 (HA)	97 (HA)
25	B	Neg	2,115	27,883	71 HA)	94 (HA)
26	B	Equivocal	209,570	> 30,000	94 (HA)	98 (HA)
27	B	Equivocal	192,190	> 30,000	91 (HA)	99 (HA)
28	B	Neg	Neg	ND	ND[e]	ND

B: breakthrough infection; E: equivocal; FU: follow-up visit; HA: high avidity; LA: low avidity; N: naïve infection; NA: not applicable; ND: not done; Neg: negative; Pos: positive.

a/ Breakthrough infection was defined as a confirmed case of measles in an individual with history of vaccination and/or positive IgG levels (> 399 mIU/mL) in acute serum at or after onset of rash (if taken within 4 days), regardless of whether post-exposure prophylaxis was given or not.
b/ Relative avidity index was calculated according to instructions by the manufacturer: HA > 60%, E 40–60%, LA < 40%.
c/ Low levels of low-avidity IgG antibodies (below the detection limit of the standard IgG assay) were detected, in Patient 2 (31%) and in Patient 7 (23%).
d/ Patient 12 had no history of vaccination against measles and no history of measles infection. This patient presented low levels of IgG (579 mIU/mL) at first sampling 5 days after onset of rash and had received post-exposure measles vaccine 7 days before onset of rash.
e/ Not done because of insufficient material.
f/ Performed at a different laboratory with the same method.

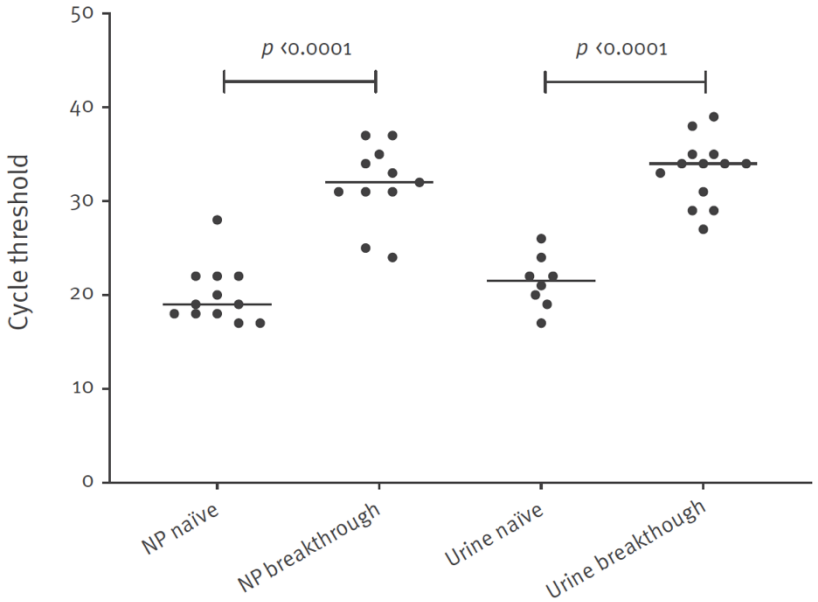

Figure 10. Cycle threshold values of measles real-time PCR in nasopharyngeal secrete and urine among patients with naïve measles infection and breakthrough infections. Sundell et al. Euro Surveill. 2019. Reprinted with permission from Eurosurveillance.

4.6 DISCUSSION PAPER III

4.6.1 Risk of onward transmission

In paper III we show that it is possible, by a fast-provisional classification, based on history of vaccination and levels of IgG at or close to onset of rash, to discern naïve from breakthrough infections. Our data indicate that the risk of onward transmission from the latter is very low. Thus, during an outbreak (in an area with a high vaccination coverage) infection control measures and contact tracing should primarily focus on naïve infections. Available reports regarding onward transmission from breakthrough infections are limited but suggest a low risk.

Rota et al. described two cases of previously vaccinated physicians who contracted measles infection during work. Both were confirmed to be breakthrough infections and although they continued to work during the symptomatic phase, also meeting unvaccinated children, no onward transmission was identified [92]. In another report by Hahné et al., describing a hospital outbreak of measles that included eight health-care workers (HCW), onward transmission was confined to one unvaccinated worker whereas the other seven (6 twice-vaccinated and 1 once-vaccinated) did not cause any secondary cases [91]. Two outbreak reports have also described onward transmission from patients with breakthrough infections, mainly to immunologically naïve but also one event of two successive breakthrough infections in one household [172, 173]. All these secondary cases occurred after prolonged close contact within households. A few additional studies have presented spread of measles from breakthrough infections [174-177]. However, laboratory and epidemiolocal details in these studies make it questionable if they in fact were breakthrough infections or not. Our study, adds important data on the features of breakthrough infections. Accordingly, we suggest that contact tracing around a case of breakthrough infection could be restricted to close household members, especially those without previous immunisation (i.e. unvaccinated children) and immunocompromised individuals.

4.6.2 Clinical presentation

The clinical presentation of naïve and breakthrough infections is discussed in paper III. Case-reports of breakthrough infections sometimes involve pronounced symptoms and may therefore not be separable from naïve infections from a clinical point of view [178]. In our study, cough was present in all naïve infections but infrequent in breakthrough infections. It is assumable that the pre-existing immunity in patients with breakthrough infections contributes to a milder clinical course and that the absence of cough limits onward transmission from these individuals. This adheres to previous reports of a milder clinical course in measles in previously immunised individuals, especially twice-vaccinated [172, 175]. Nevertheless, in our study roughly one third of the breakthrough infections fulfilled both the clinical and laboratory criteria for measles according the EU case definition. Thus, in an outbreak in an area with a high vaccination coverage, where a majority of secondary cases are likely to represent breakthrough infections, laboratory methods are needed to separate them from naïve cases. Accordingly, the suggested fast-provisional classification is therefore useful since avidity testing or plaque reduction neutralization assays, that are recommended for the confirmation of breakthrough infections, may not be accessible methods on a short notice.

4.6.3 Laboratory characteristics

Historically, the diagnosis of measles has been based on the detection of measles IgM antibodies suggestive of a primary infection. In our study almost half of the naïve cases were negative in IgM testing making it precarious to use this laboratory method alone for diagnosis. In an outbreak setting, such as the one in Gothenburg, the appearance of a rash in a (possibly) exposed individual will be the main reason for clinical evaluation, isolation and testing. Sensitivity of IgM testing may vary depending on which enzyme immunoassay being used and generally sampling in the early phase at rash onset may produce false-negative results. The sensitivity (positive IgM detection) on the day of rash onset is around 70% and upwards [179, 180]. In the case of breakthrough infections, IgM is usually not detected, but if produced it will have to compete with the presence of IgG and will likely be blocked from binding to the viral antigen in the test specimen.

The presence of measles specific IgG antibodies indicates a previous immune response after natural infection or immunisation. In our series, all breakthrough infections but two hade detectable levels of IgG in acute sera indicating a pre-existing immunity and FU samples showed a typical secondary IgG response. The detection of high-avidity IgG antibodies in these patients is suggestive of a secondary vaccine failure due to waning immunity and lack of natural boosting. It has been demonstrated that infants to vaccinated mothers have a lower level of IgG than infants to mothers with immunity after natural infection [181]. Also, the problem with primary vaccine failure (failure to seroconvert), that has been attributed to presence of maternal antibodies at time of vaccination, seems to be less common than secondary failure (where high-avidity IgG antibodies are present) [89]. In our study, two patients with breakthrough infection were negative for IgG antibodies in acute sera which could indicate a primary vaccine failure. Unfortunately, they declined follow up but both had documentation of previous immunisation and also had a mild clinical course. They had a low viral load in NP-samples, with a Ct-value of 31 and 37, respectively. It is therefore suggestable that these were breakthrough infections and presumably secondary vaccine failures due to vaccination long ago. IgG antibodies against the nucleoprotein of the virus is vital for long time immunity but also the protection against infection (post-prophylactic immunoglobulin). However, it is also worth mentioning that cellular immunity is essential for viral clearance, which is demonstrated by the recovery from measles in children with agammaglobulinemia while patients with T-cell deficiencies may develop fatal disease [182].

Nowadays, the utility of qPCR for the detection of measles RNA has made this laboratory method preferable in a suspected case, in combination with serological testing. Of the 16 breakthrough infections investigated in our study, all were positive for measles RNA in either nasopharyngeal, urine or blood samples. In coherence with a report by Seto et al., breakthrough infections had a significantly lower viral load (higher Ct value) than naïve infections [183]. Accordingly, figure 9 demonstrates semi-quantically and theoretically why the infectiousness differs significantly. In fact, two patients with naïve infections in our series ("superspreaders") transmitted measles to 24 persons, and both had a very high viral load in NP secrete combined with pronounced respiratory symptoms (cough).

4.6.4 Aspects on vaccine infections

In our study, we show that vaccine-induced infections in adults may present with pronounced symptoms and that measles RNA is detectable in clinical specimens. Although vaccine infections (also known as modified vaccine measles in literature) are well-described, our findings highlight the utility of genotyping in order to avoid confusion in an outbreak setting. Interestingly, a recent Australian study reported that measles RNA of the vaccine strain could be detected in respiratory samples by PCR in children up to 800 days after the last received vaccine dose [184]. However, since sampling is confined to symptomatic individuals during an outbreak, the risk of detecting persistent measles RNA after vaccination long-ago are unlikely.

4.6.5 Additional perspectives on breakthrough infections

More studies are needed to fully understand the nature of breakthrough infections. Plausible explanations behind the occurrence of these infections in a high-vaccination coverage setting includes waning immunity over time due to immunisation long ago, the number of received doses (some only having received one dose) and the lack of natural boosting. However, the subtype implicated in an outbreak may also be of importance. The commonly found B3 strain, and also the cause of the outbreak in Gothenburg, has been associated with higher transmissibility and might be less effectively neutralized by a vaccination-induced memory compared to other genotypes [185-187]. In contrast, a recent Italian study did not find any differences in the efficacy of vaccine-induced protection between the B3, D4 and D8 strains [188]. A continuous surveillance of circulating genotypes is mandatory to monitor the efficacy of the present vaccines used in immunisation.

4.7 RESULTS PAPER IV

4.7.1 Etiology

Of the 723 eligible patients, hospitalized with symptoms suggestive of RTI, 255 did not fulfil inclusion criteria and 248 were unwilling or unable to participate (Appendix Table 1). A total of 220 patients were included in the study. After retrieving the microbiological results of the multiplex qPCR for respiratory pathogens and the microbiological standard-of-care procedures, four etiologically different groups were created: 72 patients with viral infection, 66 with bacterial infection, 51 patients with viral/bacterial coinfection and 31 patients with unknown etiology. A total of 134 viral detections were made in 123 of the 220 patients that were included in the study (Figure 11). *S. pneumoniae, H. influenzae* and *M. pneumoniae* were the most common pathogens detected among patients with bacterial infection. In the group with viral/bacterial coinfection the predominant bacterial finding was *S. pneumoniae*, involved in 33/51 (65%) of the coinfections. The detection rate of respiratory pathogens in the four etiological groups are summarized in Table 2, paper IV.

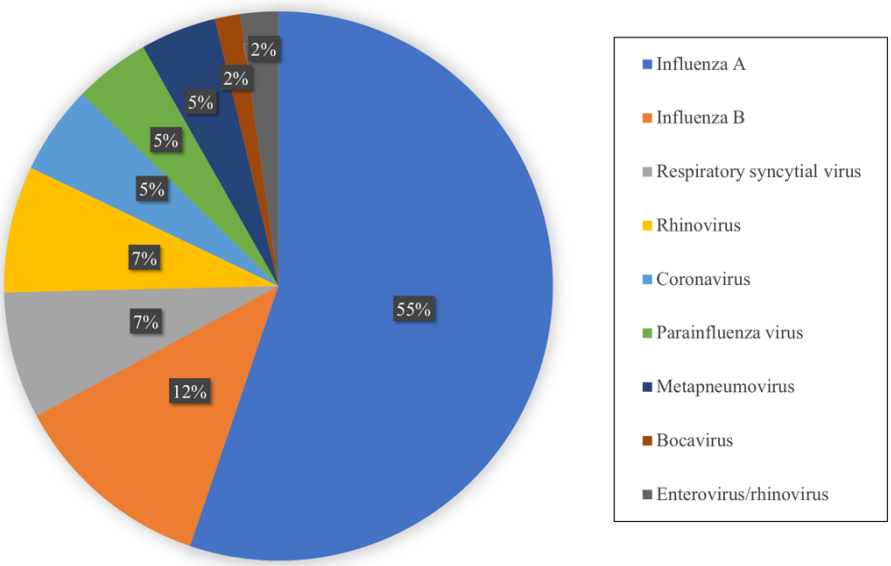

Figure 11. Distribution of the 134 viral detections made in 123 of 220 included patients in the study.

4.7.2 Factors associated with viral infection

Clinical characteristics and outcome in the group with viral infections were compared to the other etiological groups. In univariate comparisons, viral infections were associated with shorter antibiotic treatment, lower CRP level at presentation and absence of pulmonary X-ray infiltrate compared to the other etiological groups. Viral infections were also associated with higher NEWS at presentation and lower leukocyte count at presentation, compared to bacterial infections and patients with unknown etiology. The average viral infection in our study presented with a saturation of 94%, respiratory rate of 24 per minute, heart rate of 103 per minute and temperature of 38.6 °C, which reflects the elevated NEWS that were recorded upon emergency room arrival. The assessment of outcome factors showed that a shorter length of hospital stay and a lower risk of ICU admission were associated with viral infections compared to bacterial infections and patients with unknown etiology.

A multivariable model was created to distinguish factors (predictors) that were associated with viral infection. The following factors were included: Charlson comorbidity index, sex, age, immunosuppression, chronic lung disease, pulmonary X-ray infiltrate, CRB-65, NEWS, as well as CRP level, leukocyte count and non-invasive oxygen treatment, at presentation. A high NEWS score, a low CRP level at presentation and absence of pulmonary X-ray infiltrate remained independently associated with viral infection. The same associations were seen for influenza virus (IFA+IFB) when tested separately. Odds ratios and p-values are presented in Table 4, paper IV.

To further explore the findings of the multivariable model, an algorithm, based on CRP level <100 mg/L at presentation and absence of pulmonary X-ray infiltrate, was tested for the identification of viral infections. The algorithm yielded a rather poor sensitivity in identifying viral infections but the negative predictive value suggested a possibility to disclose bacterial infections. Sensitivity and specificity as well as positive and negative predictive values are shown in Table 5, paper IV.

4.7.3 Outcome

Factors associated with outcome (length of stay, mortality 30 d post-enrolment, readmission within 30 d of discharge and ICU admission) were also analysed in a separate multivariable regression model. The following factors were included: CRB-65, NEWS at presentation, Charlson comorbidity index, sex, age, immunosuppression, chronic lung disease, i.v. antibiotic treatment within 24 h, antiviral treatment within 24 h, peak C-reactive protein, peak leukocyte count, pulmonary X-ray infiltrate, non-invasive oxygen treatment at

presentation, and viral infection. Factors that remained independently associated with length of stay were immunosuppression, i.v. antibiotic treatment within 24 h of presentation and peak CRP level (shown in Table 6, paper IV). No other significant associations between the investigated factors and any of the other outcome factors were identified.

4.8 DISCUSSION PAPER IV

4.8.1 Detection rates of respiratory viruses in patients with LRTI

The main finding in the study was that syndromic testing by a multiplex PCR panel identified either viral infections or viral/bacterial coinfections in a majority of the 220 hospitalized adult patients with community-acquired LRTI. A meta-analysis that investigated the proportion of viral infections by PCR in adults with CAP reported a range from 8.6% to 56% with a pooled proportion of 25.5% in in-patients [189]. A similar study by Wu et al. also reported a combined incidence of 23% in hospitalized adults with CAP [190]. Both reports described higher detection rates of viral pathogens in lower respiratory tract samples compared to NP. In line with our study, albeit with a lower incidence, influenza virus was the most common viral pathogen in the study by Wu et al. who reported an incidence of 9%. A UK study detected influenza virus in 10% of adults hospitalized with acute respiratory infection during wintertime sampling [191]. Compared to these studies we found a higher proportion of viral pathogens (51%) and influenza virus (41%) across the entire study population. The reasons for the discrepancy are probably found in the fact that sampling occurred exclusively during wintertime in our study, when seasonal respiratory viruses are peaking in our geographical region. Also, the possibility of isolation of contagious patients (especially suspected influenza cases) at the Department of Infectious Diseases may also have created a selection bias towards more influenza patients.

4.8.2 Comments on different viral pathogens

As discussed earlier, detection of respiratory viruses in healthy asymptomatic adults are uncommon. Thus, one would assume that a positive detection in an adult patient with ongoing symptoms of LRTI is clinically relevant. Accordingly, influenza virus and other non-influenza viruses such as PIV, RSV and HMPV are often considered to be the causative agent when detected, especially in immunocompromised. We found these non-influenza viruses to

be a rare cause of hospitalization in our study, mainly affecting immunocompromised, which serves as a reminder of the disease burden they impose on these patients. However, they seem to be a rare cause of hospitalization in otherwise healthy adults in our setting, and are probably in general more associated with a self-limiting mild RTI. Nevertheless, these viruses may be implicated in viral/bacterial coinfections as seen in our study and in a study by Crotty et al [192]. They reported a higher incidence of these pathogens in hospitalized patients but the high proportion of severely immunocompromised individuals in that study may explain the diverging results.

Paper I and paper II showed that HRV was prevalent across all seasons and the predominant pathogen when respiratory viruses were detected in asymptomatic adults. However, the importance HRV have arguably been underestimated among clinicians for several years. More recent studies have begun to recognize this pathogen in adults with CAP [191, 193, 194] and also reported HRV to be the most frequently detected respiratory virus in these patients [162, 191]. In our study HRV was uncommon in patients with viral infections but more frequently detected among viral/bacterial coinfections. Given the previously discussed features of HRV, it is possible that some detections of this pathogen in our series may be unrelated to the actual cause of hospitalization. Our findings also suggest that HRV alone rarely causes respiratory infections that requires hospital care unless other underlying conditions such as COPD or immunosuppression are present. Nevertheless, HRV may be associated with bacterial superinfections as also been described by others [194]. It is likely that future studies will further discover the burden of disease that is related to this pathogen.

4.8.3 Viral or bacterial infection?

The ability to distinguish viral from bacterial infection in patients with LRTI and CAP could aid antibiotic guidance [195]. However, this remains challenging in a clinical setting and antibiotic overuse is still common in viral infections as seen in our study and others [191]. To make matters worse, our data, along with others, clearly show that viral/bacterial coinfections are common in adults with CAP. Coinfections have also been reported to contribute to a more severe clinical course, highlighting the need of antibiotic treatment in some of these patients [162, 189, 196]. CRP and pulmonary chest X-ray may still offer some guidance in order discern viral infections from other etiological causes where antibiotic treatment is needed [197, 198]. Our algorithm, based on CRP at presentation and absence of pulmonary X-ray infiltrate had a relatively poor sensitivity and positive predictive value. This is consistent with other reports [199]. Nevertheless, from a clinical point of view,

this uncontroversial algorithm might be more accurate in identifying patients in need of antibiotic treatment.

In our study, the most frequent pathogens in viral/bacterial coinfections were influenza virus and *S. pneumoniae,* a common combination and well described [200, 201]. *H. influenzae* was also a regularly detected co-pathogen in viral/bacterial infections, and it is likely that this is an important bacterium in adults with CAP. The detection rate of this pathogen was also significantly higher among symptomatic subjects in comparison with the asymptomatic cohort in paper II. In the previous literature, the frequency of *H. influenzae* in CAP are varying, however [202-204]. The lack of conventional cultures in our study precludes any strong conclusion regarding the importance of this bacteria in patients with LRTI/CAP and future studies are needed to further elucidate how to interpret PCR-based detection of these bacterial pathogens in the upper airways. Also, other bacteria such as *S. aureus* and *S. pyogenes* may have been underestimated in our study due to the lack of complete sample sets (cultures) in many of the patients.

4.8.4 Clinical presentation and outcome

Immunosuppression has previously been associated with increased in-hospital mortality in patients with CAP but the limited number of deaths in our study hampers any comparison [192]. On the other hand, we found immuno-suppression to be the only patient-related factor that was associated with length of stay. A high level of peak CRP and initiation iv antibiotic treatment at presentation were also associated with increased length of stay. This serves as an explanation as to why bacterial infections and patients with unknown etiology were associated with a longer hospital stay compared to viral infections and viral/bacterial co-infections. A finding that stands in contrast to other reports [196, 205]. Few ICU admissions and a high proportion of viral infections and viral/bacterial infections (with a relatively mild clinical course) in our study may account for the discrepancy.

We found that a high NEWS at presentation was associated with viral infections. A high score could likely affect the clinician's decisions in an emergency setting and contribute to an increased likelihood of hospitalization and initiation of broad-spectrum antibiotic treatment. Increased knowledge of the clinical presentation of viral infections combined with an early access to point-of-care testing for respiratory viruses with a rapid turn-over, would likely be beneficial in identifying patients with pure viral infections.

4.9 ADDITIONAL RESULTS PAPER IV

4.9.1 Cap versus non-CAP

In paper IV, we analysed four different etiological groups of patients hospitalized with LRTI, with emphasis on clinical characteristics and outcome in patients with viral infections. However, based on the microbiological methods that were used in the study, primarily focusing on PCR-based detection in NP-samples, one could argue if the etiological classifications truly reflected the actual causative agent. In order to address this issue further, the included patients were instead stratified according to the criteria for CAP set by the British Thoracic Society.

Based on these criteria a sub analysis was performed of the 189 patients who underwent pulmonary X-ray at presentation. A total of 123 patients fulfilled CAP criteria by having a novel pulmonary X-ray infiltrate whereas 66 patients had no novel finding (non-CAP). The basic microbiological findings in each group are presented in Table 10. Detection of viral pathogens as well as detection of influenza virus, was significantly associated with non-CAP patients. Several clinical factors were significantly associated with CAP, especially the proportion of patients receiving iv antibiotic treatment and the level of CRP and leukocyte count at presentation (Table 11). CAP was also associated with longer hospital stay and increased risk of ICU admission. Interestingly, NEWS at presentation (that was associated with viral infection in paper IV) did not differ between the groups.

A multivariable regression was performed to further analyse if any of the commonly detected pathogens or factors at presentation were independently associated with CAP. The following factors were analysed: detection of influenza virus, any detection of *S. pneumoniae*, any detection of *H. influenzae*, Charlson comorbidity index, sex, age, immunosuppression, chronic lung disease, CRB-65, NEWS, CRP level, leukocyte count and non-invasive oxygen treatment at presentation. We found that any detection of *S. pneumoniae*, a high level of CRP and need of oxygen treatment at presentation were independently associated with CAP (Table 12).

Factors associated with outcome for the entire sub population (n=189) were also analysed in a second multivariable regression model. The following factors were tested for association with outcome: CRB-65, NEWS at presentation, Charlson comorbidity index, sex, age, immunosuppression, chronic lung disease, i.v. antibiotic treatment within 24 h, antiviral treatment

within 24 h, peak C-reactive protein, peak leukocyte count, non-invasive oxygen treatment at presentation, and CAP. In line with the findings in paper IV, immunosuppression, i.v. antibiotic treatment within 24 h of presentation and peak CRP remained independently associated with a longer hospital stay. No other significant associations between the investigated factors and outcome were identified.

Table 10. *Summary of the basic microbiological findings in the sub analysis of patients with CAP and patients not fulfilling CAP-criteria.*

Pathogens	**Patients with CAP** *(n=123)*	**Patients not fulfilling CAP-criteria** *(n=66)*	***p-value***[1]
	n (%)	*n (%)*	
Any pathogen detected	100 (81)	60 (91)	0.08
Any virus detection	45 (37)	53 (80)	<0.0001
Any bacterial detection	80 (65)	24 (36)	0.0002
Influenza virus detected	26 (21)	42 (63)	<0.0001
Only influenza virus detected	14 (11)	29 (44)	<0.0001
Pure viral detection	20 (16)	36 (55)	<0.0001
Pure bacterial detection	55 (45)	7 (11)	<0.0001
Viral bacterial co-infection	25 (20)	17 (26)	0.4
Unknown etiology	23 (19)	6 (9)	0.08
Any detection of *S. pneumoniae*	35 (28)	12 (18)	0.1
Only by PCR	22 (18)	8 (12)	0.3
Only by SOC	8 (7)	2 (3)	0.5
By both PCR and SOC	5 (4)	2 (3)	1
Any detection of *H. influenzae*	24 (20)	10 (15)	0.6
Only by PCR	22 (18)	5 (8)	0.08
Only by SOC	1 (1)	2 (3)	NC
By both PCR and SOC	1 (1)	3 (5)	NC

Abbreviations: CAP=community acquired pneumonia, SOC=standard-of-care procedures
[1] *Pearson chi-square test or Fisher´s exact test when appropriate if not stated otherwise*

Table 11. *Clinical and laboratory characteristics in patients with CAP compared with patients not fulfilling CAP criteria.*

Characteristics	**Patients with CAP** (***n=123***)	**Patients not fulfilling CAP-criteria** (***n=66***)	***p value***[1]
Clinical data			
NEWS *median, (IQR)*	4 (3-7)	5 (2-7)	0.4[2]
CRB-65 >1	12 (10%)	5 (8%)	0.8
Antimicrobial therapy			
Antibiotic treatment at any time	119 (97%)	54 (82%)	0.0004
Empiric iv antibiotic treatment within 24 h of presentation	105 (85%)	41 (62%)	0.0003
Total days of antibiotic treatment *median, (IQR)*	8 (7-10)	7 (1-9)	<0.0001[2]
Antiviral treatment within 24 h of presentation	31 (25%)	42 (64%)	<0.0001
Laboratory			
CRP (mg/L) at presentation *median, (IQR)*	150 (68-290)	48 (24-86)	<0.0001[2]
Peak CRP (mg/L) *median, (IQR)*	200 (120-310)	81 (41-140)	<0.0001[2]
Leukocyte count (10^9/L) at presentation *median, (IQR)*	10 (6-13)	8 (5-10)	0.01[2]
Leukocyte count (10^9/L) at peak *median, (IQR)*	10 (7-14)	8 (5-11)	0.008[2]
Respiratory support			
Non-invasive oxygen treatment	81 (66%)	28 (42%)	0.002
Total days of oxygen treatment *median, (IQR)*	2 (0-5)	0 (0-2)	<0.0001[2]
Outcome			
Length of stay *median, (IQR)*	5 (4-9)	4 (3-6)	0.01[2]
Death <30 d post enrolment	2 (2%)	1 (2%)	NC
Readmission within 30 d of discharge	14 (11%)	8 (12%)	0.9
ICU admission	15 (12%)	1 (2%)	0.01

[1] Pearson chi-square test or Fisher´s exact test when appropriate if not stated otherwise
[2] Mann-Whitney test
Abbreviations: IQR= inter quartile range, NC=not calculated, CAP=community-acquired pneumonia

Table 12. *Multivariable logistic regression analysis of factors associated with community acquired pneumonia.*

Predictors[1]	odds ratio (95% CI)	*p*-value
Detection of influenza virus	0.2 (0.08-0.5)	0.0002
Any detection of *Streptococcus pneumoniae*[2]	3.1 (1.2-8.3)	0.02
Chronic lung disease	0.3 (0.1-0.7)	0.006
C-reactive protein (mg/L) at presentation *(+25 mg/L)*	1.2 (1.1-1.4)	0.001
Oxygen treatment at presentation	3.5 (1.5-8.2)	0.004

[1]*Only associations with p value below 0.05 are presented*
[2]*Regardless if detected by PCR or standard-of-care procedures*

4.10 ADDITIONAL DISCUSSION PAPER IV

4.10.1 Aspects on CAP and non-CAP patients in our cohort

Influenza was clearly associated with non-CAP patients and detected in almost half of the patients in this group. Consistent with this, the multivariable logistic model showed that detection of influenza virus was independently associated with non-CAP infections. Nevertheless, in around 10% of the patients with CAP, influenza was the only detected pathogen. Although samples in our study were collected during the time of the seasonal flu, it underlines that influenza alone is a common cause of CAP in adults. Our detected frequency is similar to a US study of hospitalized adults with CAP, who identified influenza in 6% of the patients [203]. Also, roughly one-third of the CAP-patients were positive for a respiratory virus stressing the importance of viral testing in this patient group. The presence of chronic lung condition was independently associated with non-CAP infection. The high proportion of influenza patients with underlying medical conditions, and isolated at our unit, are likely to account for this finding. However, it should not be interpreted as patients with chronic lung condition are at lower risk of developing CAP. Lastly, unknown etiology were common in patients with CAP suggesting that many of these were undiagnosed bacterial infections.

4.10.2 *S. pneumoniae* and *H. influenzae* detected by PCR

Interestingly, when conventional cultures were excluded, the proportion of PCR-based detections of *S. pneumoniae* and *H. influenzae* did not differ significantly between the two cohorts. Accordingly, PCR positive detections of *S. pneumoniae* did not remain independently associated with CAP in the multivariable logistic model. This highlights the need for future studies regarding the clinical relevance of PCR-based detection of *S. pneumoniae* in NP-samples in adults with LRTI and CAP. However, it is noteworthy that a rather large proportion of patients with CAP were PCR positive for *S. pneumoniae* and *H. influenzae*, especially when compared to the frequencies that were found among asymptomatic and symptomatic subjects in paper II. This could imply that detection of these bacteria by PCR in NP samples may be indicative of etiological diagnosis in patients fulfilling CAP criteria, regardless if a respiratory virus is detected or not. In non-CAP patients the detection of *S. pneumoniae* and *H. influenzae* by PCR was mainly associated with viral/bacterial coinfections. One could speculate, that the PCR-based detections of these bacteria in this cohort might be due to a bacterial superinfection but also an asymptomatic carriage during ongoing respiratory viral infection. Questions concerning the true clinical relevance of the positive bacterial findings in this cohort cannot be answered by our data and warrants further studies.

Previous PCR-based studies have yielded higher detection rates of pneumococci in NP samples in both symptomatic and asymptomatic individuals compared to cultures [95-99]. The risk of cross reaction with other streptococci seems to be low when the Spn9802 fragment is targeted by the PCR (as done in our study). Quantitative PCR on sputum has been shown to be superior to NP samples in identifying pneumococcal etiology in adults [206]. Nevertheless, a recent study investigating the utility of oropharyngeal qPCR, for the detection of *S. pneumoniae* and *H. influenzae* in patients with CAP, revealed a relatively high sensitivity and specificity in identifying the cause of pneumonia, at least in conjunction with other methods [207]. A study by Strålin et al. compared cultures and multiplex PCR on sputum, NP swabs and NP aspirates in adults with CAP. They found a low detection rate of *S. pneumoniae* and *H. influenzae* by PCR in 113 controls without respiratory symptoms which is consistent with our findings in paper II. They also reported that NP-samples could aid etiologic diagnosis in patients with pneumonia [208]. Furthermore, a recent study looking at respiratory viral infections in patient with suspected sepsis, found a strong correlation between the detection of these bacteria in NP-samples (culture) and the presence of a novel infiltrate suggestive of pneumonia while no detections were made in non-respiratory patients [209]. In summary, PCR-based detections of these pathogens in NP-

samples are useful in a clinical setting and could aid etiologic diagnosis, especially in adults with CAP. Nevertheless, more studies are needed to fully understand the usefulness of this sensitive molecular method in adults with ongoing symptoms of RTI, especially since it remains a challenge to obtain lower respiratory tract samples in a clinical setting.

5 CONCLUSIONS

- The seasonal variation of influenza virus and other enveloped respiratory viruses are strongly correlated with outdoor temperature and absolute humidity in a temperate climate. A sudden drop in outdoor temperature (and absolute humidity) may trigger the onset of the annual influenza epidemic in our region, suggestively by facilitating aerosol spread in dry air. Human rhinovirus is prevalent across all seasons, independent of outdoor temperature and absolute humidity, suggesting other routes of transmission than through aerosol for this pathogen (i.e. contact and droplet).

- In adults without ongoing symptoms of respiratory tract infection, detection of respiratory viruses by PCR in nasopharyngeal samples is uncommon (<5%). Human rhinovirus is the most common viral respiratory pathogen detected among asymptomatic adults. Thus, a positive viral detection by PCR in a symptomatic patient is likely to be relevant for etiologic diagnosis.

- Breakthrough infections are common during an outbreak of measles in an area with a high vaccination coverage. Viral load in nasopharyngeal samples is higher in patients with naïve than in breakthrough infections. Onward transmission is likely confined to naïve infections. A fast-provisional classification, based on history of vaccination and presence of IgG antibodies, can identify breakthrough infections and guide contact-tracing in an outbreak setting.

- A multiplex PCR panel identified viral infections or viral/bacterial coinfections in a majority of hospitalized adults with community-acquired LRTI. To distinguish viral infections from bacterial infections, based on clinical presentation and routine laboratory findings alone, remains challenging. CRB-65 and NEWS add limited value while radiology and CRP may be helpful in identifying patients in need of antibiotic treatment.

6 FUTURE PERSPECTIVES

The results presented in this thesis have added a little piece to a very large and complex puzzle. The heterogeneity and abundance of respiratory viral pathogens will ensure that they will continuously impose a threat to us, both individually and globally. The routes of transmission are difficult to effectively control, especially with the travelling patterns of the modern society. Respiratory viruses will gradually evolve both genetically and antigenically, particularly through human-animal interaction, thereby finding new hosts to infect. This is highlighted in the ongoing outbreak of COVID-19 in China, that has spread rapidly and already surpassed SARS in terms of mortality. Also, in the wake of climate change, the seasonality of respiratory viruses may shift gradually leading to new epidemiological patterns of these pathogens. Furthermore, vaccine scepticism may pave the way for the resurgence of old acquaintances such as measles that now again is affecting more and more people instead of being eliminated from parts of the world. Lastly, the ever-growing problem with antibiotic resistance warrants the need for improved microbiological techniques that pin-point patients in need of antimicrobial treatment and those who don't. All these future perspectives combined with the findings presented in this thesis suggest continuous research within the following fields.

More studies are needed to further explore the impact of temperature and absolute humidity on aerosol dynamics, viral transmission and seasonality. Emphasizing on the interplay between these two parameters is of importance and whether they should be considered as separate factors or one single entity. In light of the growing epidemic of COVID-19, there is a need to more fully unravel which routes of transmission that are primarily implicated in the spread of different respiratory viral pathogens. More so, this field needs more experimental studies including human subjects, to further explore the dynamic processes of viral transmission, in order to comprehend when and why a specific virus may expand beyond traditional contact and droplet transmission and suddenly become airborne.

Continuous research of the utility and usefulness of PCR detection in NP samples is needed to further explore how viral load, as estimated by Ct values, corresponds to infectivity and clinical presentation (asymptomatic vs symptomatic infection). In addition, more studies are needed to assess the pros and cons of point-of-care testing using novel broad multiplex PCR panels in a clinical setting, especially in patients with LRTI and CAP.

More prospective studies are also warranted to assess the clinical relevance of PCR-based detection of pathogens in the upper airways. Especially investigations that more distinctively discern accurate etiological identification of disease-causing pathogens in the airways from detections that are not relevant or as of yet, only presumed to be relevant.

The resurgence of measles in many parts of the world requires an increased awareness of breakthrough infections. Future studies, on larger patient materials, will hopefully confirm our findings that breakthrough infections can be safely identified early on through measurement of IgG antibodies in acute sera combined with history of vaccination. At present, there is enough data to update regional and national guidelines on the clinical and laboratory characteristics of breakthrough infections in order to guide contact tracing and infections control in future outbreaks.

Lastly, an increased monitoring and surveillance of circulating respiratory viral strains and genotypes are needed for the early identification of novel pathogens that have the potential to cause new pandemics with significant morbidity and mortality.

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APPENDIX

Appendix Table 1. *Comparison of the 220 subjects included in the study and the 248 who were not included.*

Characteristics	**Not included patients[1] (n=248)**	**Patients in the study (n=220)**	***p*-value[2]**
Age *Yrs, (IQR*)	64 (46-76)	61 (43-75)	0.4[3]
Females (%)	120 (48)	130 (59)	0.02
Length of stay, *(IQR)*	5 (2-10)	5 (3-7)	0.6[3]
Mortality 30 days post enrolment (%)	13 (5)	4 (2)	0.08
Diagnosis of any type of respiratory tract infection at discharge (%)	227 (92)	NA	

[1] language difficulties n=91, in mechanical ventilator or sedated n=30, unwilling n=55, other reasons n=72
[2] Pearson chi-square test or Fisher's Exact test when appropriate
[3] Mann-Whitney test