On the Diagnosis and Management of Viral Respiratory Infections

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To Susan

" I love the doctors - they are dears;
But must they spend such years and years
Investigating such a lot
Of illness which no one's got,
When everybody, young and old,
Is frantic with the common cold?
And I will eat my only hat
If they know anything of that!"

*Herbert AP. The common cold. In: Look back and laugh. London: Methuen, 1**960:** 115–17.*

Abstract

Acute respiratory tract infections (ARTIs), are the most common infections in man, and represent a major global health burden. Viruses, most often causing a mild and resolving disease, yet with substantial morbidity and high costs for society, mainly cause upper respiratory tract infections. 70% of all infections in primary care in Sweden are due to ARTIs. Lower respiratory infections on the other hand constitute the third leading cause of death worldwide, mainly in children <5 years of age in resource poor settings. Distinguishing virus from bacteria can be difficult, and often lead to an over-prescription of antibiotics. Modern molecular based diagnostic methods have increased the possibility of an etiologic diagnosis of ARTIs significantly. This thesis aims to evaluate the use of a multiplex real time PCR assay targeting 13 respiratory viruses and two bacteria, from a clinical perspective.

In paper I, a retrospective study of 954 nasopharyngeal samples, the PCR assay, which is based on automated specimen extraction and multiplex amplification, is described. Detection rate was 48%. Streamlined testing and cost limitation (€ 33 per sample) along with high accuracy and prompt result delivery, is key to successful implementation of broad molecular testing.

Paper II evaluates in a prospective study of 209 adults with ARTI in primary care, and 100 asymptomatic controls, the impact duration of symptoms have on detection rate. Overall positive yield was 43% in patients and 2% in controls, with a significantly higher detection rate in patients with < 6 days duration of symptoms (51%) compared to ≥ 7 days (30%, $p<0.01$).

Having access to the PCR assay significantly reduced antibiotic prescription, in a prospective study (paper III) of 406 adults with ARTI. Patients receiving a result within 48 hours were prescribed antibiotics in 4.5% (n=9 of 202), compared to 12.3% (n=25 of 204, $p=0.005$) in the delayed result group.

The diagnostic yield in paper IV, a retrospective study of 8753 patients of all ages during 36 consecutive months, was significantly higher during winter (54.7%) than in summer (31.1%, $p<0.001$), and in children (61.5%) compared with adults (30.5%, $p<0.001$). Rhinovirus was the most frequently found virus (32.5%), independent of season, and displayed a high genetic variability across seasons.

The findings of this thesis support the implementation of similar methods in routine clinical care.

Keywords: Respiratory virus, Respiratory tract infection, Real-time PCR, Multiplex PCR, Antibiotic use.

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SAMMANFATTNING PÅ SVENSKA

När min två år gamla son får sin fjärde förkylning på lika många månader, och hans hosta enträget pockar på vår uppmärksamhet nattetid, då känner jag blicken bränna i nacken från min fru: - Är det inte dags för en kur antibiotika nu? Han blev ju bra förra gången han fick antibiotika??

Luftvägsinfektioner kan orsakas av både virus och bakterier, och utgör sannolikt den vanligaste gruppen av infektioner av alla hos människan. De flesta av dessa ger upphov till en relativt mild och självläkande infektion som drabbar den övre delen av luftvägarna (näsa, munhåla, hals, bihålor, öron), och som i merparten av fallen orsakas av ett virus. Infektioner i de nedre delarna av luftvägarna (luftrören och lungorna) kan orsaka allvarlig sjukdom och innefattar ofta bakterier som orsakande organism. Både virus och bakterier kan dock infektera hela luftvägarna.

Det kan ibland vara svårt att skilja mellan virus och bakterier, även för den tränade läkaren, då bedömningen framförallt vilar på patientens symtombild och den kroppsliga undersökningen av patienten. Tyvärr finns inget enkelt blodprov eller dylikt, som med stor säkerhet kan särskilja virus från bakterier. Detta medför bland annat att antibiotika (som endast har effekt på bakterier och inte på virus) ofta ordineras i onödan. Denna överförskrivning av antibiotika bidrar till stort lidande i form av biverkningar, allergiska reaktioner samt följdsjukdomar. Dessutom tilltar bakteriernas motståndskraft mot antibiotika när dessa används i onödan.

Modern diagnostik, som baseras på att arvsmassa från virus och bakterier påvisas, har ökat möjligheten till rätt diagnos. Denna avhandling utvärderar den kliniska nyttan och användningen av en sådan diagnostisk metod, s.k. PCR-teknik, som analyserar 13 stycken virus och två stycken bakterier. Prov som analyserat i samtliga delarbeten är tagna från området bakom näsan, samt från bakre svalgväggen.

I delarbete I beskrivs metoden. I 48 % av 954 analyserade prover fann man ett virus eller bakterie. Rationalisering på laboratoriet, ett relativt lågt kundpris (€ 33 per prov) samt ett snabbt svar till patientens läkare är av stor vikt om denna typ av diagnostik skall införas på bred front.

I delarbete II undersöktes 209 vuxna patienter som sökt vård på vårdcentral för en akut luftvägsinfektion, i en framåtblickande (prospektiv) studie. Tiden som förlupit från att symtomen startade till dess att provtagning gjordes (s.k. symtomduration) visade sig vara viktig för möjligheten att påvisa virus.

Patienter som varit sjuka i upp till 6 dagar hade en signifikant högre andel positiva fynd (51 %) jämfört med patienter med 7 eller flera dagars sjukdomsduration (30 %, $p<0.01$).

Tillgång till snabb diagnostik med den utvärderade PCR metoden minskade signifikant antibiotika förskrivningen in en grupp av 406 vuxna patienter med luftvägssymtom, där hälften slumpmässigt utvaldes att få svar inom 48 timmar och hälften fick svar 10 dagar efter provtagning (delarbete III). 9 patienter (4.5%) i snabbsvarsgruppen och 25 patienter (12.3%, $p=0.005$) i fördröjt svarsgruppen erhöll antibiotika.

I delarbete IV analyserades 8753 prover i efterhand, tagna från patienter i alla åldrar med symtom på luftvägsinfektion. Studieperioden innefattade 36 månader i följd under 2006-2009. Andel positiva fynd var signifikant högre under sommar halvåret (54.7 %) än under vinter halvåret (31.1 %, $p<0,001$), och bland barn (61.5 %) jämfört med vuxna (30.5 %, $p<0,001$). Rhinovirus var det vanligaste fyndet i positiva prover (32.5 %), oberoende av säsong. Detta virus uppvisade en hög genetisk variation (dvs. med många olika sub-typer av virus) genomgående under alla årstider.

Fynden i denna avhandling stödjer införandet av liknande diagnostiska metoder i klinisk rutin.

LIST OF PAPERS

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

I. Brittain-Long R, Nord S, Olofsson S, Westin J, Andersson L-M, Lindh M. **Multiplex real-time PCR for detection of respiratory tract infections.** *Journal of Clinical Virology 41 (2008) 53–56*

II. Brittain-Long R, Westin J, Olofsson S, Lindh M, Andersson L-M. **Prospective evaluation of a novel multiplex real-time PCR assay for detection of fifteen respiratory pathogens – Duration of symptoms significantly affects detection rate.** *Journal of Clinical Virology 47 (2010) 263–267.*

III. Brittain-Long R, Westin J, Olofsson S, Lindh M, Andersson L-M. **The use of a multiplex real time-PCR method targeting thirteen viruses – impact on antibiotic prescription rate in a prospective study.** *In manuscript.*

IV. Brittain-Long R. Andersson L-M, Lindh M. Westin J. **Seasonal variations influence diagnostic yield of a multiplex PCR assay targeting 13 respiratory viruses.** *In manuscript.*

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ABBREVIATIONS

AdV	Adenovirus
ACAS	Acute Community Acquired Sinusitis
AOM	Acute Otitis Media
ARTI	Acute Respiratory Tract Infection
CAP	Community Acquired Pneumonia
CoV	Coronavirus
CRP	C-Reactive Protein
Ct-value	Cycle Threshold value
DFA	Direct Fluorescent Antibody test
dsDNA	Double stranded DNA
EIA	Enzyme Immuno Assay
ELISA	Enzyme-linked Immunosorbent Assay
EV	Enterovirus
HMPV	Human Metapneumovirus
HRV	Human Rhinovirus
IF	Immunoflourescence
IfA	Influenza A Virus
IfB	Influenza B Virus
LRTI	Lower Respiratory Tract Infection
NAAT	Nucleic Acid Amplification Test
NPH	Nasopharyngeal
PCR	Polymerase Chain Reaction
PCT	Procalcitonin
PIV	Para Influenza Virus
qPCR	Quantitative Real time PCR
RSV	Respiratory Syncytial Virus
RTI	Respiratory Tract Infection
ssDNA	Single stranded DNA
URTI	Upper Respiratory Tract Infection

1 INTRODUCTION

Respiratory tract infections (RTIs) are the most common infections of all in humans. In terms of mortality, lower RTIs are the third leading cause of death worldwide, in all categories and all age groups (1). It is however children below the age of five, in low-income countries that suffers the most from this deadly disease entity. In terms of morbidity acute RTIs (ARTIs) still make up a considerable bulk of health care visits even in middle- and high-income countries. In Sweden 70% of all infectious disease complaints in primary care are due to ARTIs (2) and up to one third of all consultations are because of ARTIs (3).

These infections are mainly caused by bacteria and viruses, of which the latter dominate in the upper respiratory tract. Viruses are however also common in the lower respiratory tract as was shown in a study of LRTIs in primary care in England, which detected respiratory viruses in 63% of cases (4), and several prospective studies on community acquired pneumonia (CAP) has detected viruses as the second most common pathogen after streptococcus pneumoniae (5).

The costs associated with ARTIs are staggering. For non-influenza viral ARTIs in the United States alone, the economic impact has been estimated to USD 40 billion annually (6).

It can be difficult on clinical grounds to discriminate between ARTIs caused by viruses and those caused by bacteria, and the differentiation is important since therapeutic options differ significantly. Bacterial infections of the respiratory tract should in some circumstances be treated with antibiotics, which in fact can be life saving, but antibiotics have no effect on viruses. This has led to a considerable overuse of antibiotics in the treatment of ARTIs (7). Acute bronchitis serves as an illustrative example. The recommended treatment of acute bronchitis (which is caused by viruses in the majority of cases) in immunocompetent adults does not include antibiotics (8-10). This is valid also in low-income countries with a high HIV prevalence (11). Nevertheless, antibiotics are prescribed for the diagnosis of acute bronchitis at high rates. In the UK study by Creer et al., although a virus was found in 63% of cases of acute bronchitis and bacteria in 26% of cases, antibiotics were prescribed in 64% of patients (4). In Sweden a reported rate of antibiotics prescribed for bronchitis of 50% has been reported (2), and in the United States 59% (12).

Unnecessary use of antibiotics has major implications, not only for health economics, but also even more importantly for the development of bacterial resistance (13, 14), as well as for the individual patient in terms of adverse events such as allergic reactions and antibiotic-associated diarrhoea (15, 16).

The development of modern molecular based diagnostic tools for ARTIs, and in particular viral ARTIs, such as nucleic acid amplification tests (NAATs, the term includes any test that directly detects the genetic material of the infecting organism) have considerably increased the possibility of an etiologic diagnosis of ARTIs. Furthermore, these tests have has prompted the discovery of novel viruses causing respiratory disease in humans, such as human metapneumovirus (HMPV) (17), SARS-associated coronavirus (SARS-CoV) (18) and two new coronaviruses, CoV NL63 (CoV-NL63) (19) and CoV-HKU1 (20). Other recently discovered viruses found in the respiratory tract of humans, but so far with uncertain significance as pathogens, are human bocavirus (hBoV) (21), polyomavirus KI (KIPyV) (22) and WU virus (WUV) (23). There is in fact increasing support for human bocavirus as a human pathogen, mainly affecting children and most often in conjunction with another respiratory virus (24-26), but for KIPyV and WUV the evidence for a causative link to respiratory disease in humans is low (27, 28)

Detection of hitherto unknown viruses by these highly sensitive methods may result in a proven association between the agent and a specific disease, but causality is often more difficult to prove. Strictly speaking Koch's postulates formulated in 1890 which dictates that; (1) the agent is present in every case of a disease, (2) it is specific for that disease, (3) it can be propagated in culture, and (4) can be inoculated into a naive host to cause the same disease, should be applied. A more modern version of Koch's postulates, which has been adapted to viruses, was suggested by Fredericks and Relman in 1996 (29) and states in brief that:

1. The organism is detected with significantly higher prevalence than in control subjects
2. The organism causes disease in an animal model and can subsequently be detected
3. A specific immune response against the virus can be detected in the host

Several problems still remain if these criteria are applied to prove causality, for example; there may not be a suitable animal model and the result of infection may vary depending on age, genetic background or previous exposure to the virus.

Nevertheless, NAATs played a crucial role in molecular characterization and rapid response measures to severe health threats like the SARS epidemic in 2003 and the 2009 H1N1 pandemic.

A rapid etiological diagnosis of ARTIs provides proper treatment options for viral and bacterial infections, a better estimate of the prognosis of the disease, length of stay in hospital or absence from work, school or day-care, and for infection control (30). It has been suggested that the use of NAATs or other rapid diagnostic tools, for detection of respiratory viruses, could be a useful tool in reducing unnecessary antibiotic prescriptions (12, 31, 32). However, if these assays are to be implemented, proper validation and standardization of multiplex real-time PCR methods need to be applied.
Furthermore, the epidemiology and seasonal distribution of the viruses that cause the majority of respiratory tract infections in humans, is important for the treating physician to understand in order to better diagnose and manage these infections.

1.1 Diagnostic laboratory methods for viral respiratory infections

Diagnosing virus in the respiratory tract of humans is based on four principally different methods, the first three detects the virus or parts of the virus, and the fourth method detects the immune response elicited by the infected individual:

1. Virus isolation
2. Antigen detection
3. Genome detection
4. Serology

1.1.1 Virus isolation

Virus isolation in cell culture constituted the basis of diagnostic virology in the early days of this discipline and has been the golden standard to which other methods have been compared. This labour-intense method includes inoculating a patient sample that contains the suspected virus on a cell culture (monkey-kidney cells or human fibroblasts are commonly used), which is susceptible to several viruses. Frequently, several cell-lines are needed. The cytopathogenic effect (swelling or destruction of cells), specific to each virus, on the cell culture is then recognized as a positive sample. One advantage is

that a living cell strain is acquired, which can be analysed further. The process is relatively slow, normally days or weeks are required before a result can be provided to the treating physician, and is therefore often too late to guide the treatment of the patient (33).

1.1.2 Antigen detection

Antigen detection methods, such as enzyme-linked immunosorbent assay (ELISA), are carried out by first inoculating the patient sample on a surface. The surface can either be pre-coated with an antibody that captures the virus antigen, or the antigen is directly adsorbed to the surface. The next step involves adding a specific enzyme-linked antibody that forms an antigen-antibody complex. Finally a substance that the enzyme can convert into a detectable signal (often a fluorescent signal) is added. By using monoclonal antibodies a high degree of specificity can be accomplished. To increase the sensitivity various enzymatic enhancement steps, so called enzyme immunoassays (EIA) can be applied.

In immunoflourescence (IF) the localization of virus proteins to different parts of the infected cell increases the specificity. Direct fluorescent antibody tests (DFA) use antibodies that are tagged with fluorescent dye and are available for rapid antigen testing. Commercial kits for Influenza A and RSV are widely used and has the advantage of being a rapid bed-side test, but lack in sensitivity compared to virus isolation and genome detection (34, 35). Another advantage with antigen detection is that non-viable viruses can be analysed, which may be important when samples need a long transportation to the laboratory.

1.1.3 Genome detection

Nucleic acid amplification tests have had a major impact on diagnostic virology and the detection of respiratory viruses, in that sensitivity has increased considerable and viruses that were previously difficult to demonstrate with conventional methods can be detected in patient samples.

The polymerase chain reaction (PCR) method was first described by Kary Mullis in 1983 (36) and awarded him the Nobel Prize in Chemistry in 1993. PCR is a process that amplifies a single or a few copies of DNA into millions of copies of a particular DNA sequence (from a virus in this case). The method relies on thermal cycling with repeated heating and cooling, resulting in DNA melting and enzymatic replication. Normally 30-40 cycles are

carried out. Each new copy of the DNA strand acts as a template for the next replication cycle, setting in motion a chain reaction, which results in an exponential amplification.

A specific heat stable polymerase, such as the one used in the PCR assay evaluated in this thesis, is the Taq polymerase. It was isolated in 1976 from the thermophilic bacteria *Thermus aquaticus (37)* that thrives in hot springs at temperatures of 70°C, and eliminates the need for adding new enzymes after each cycle of the PCR process, thereby speeding up the process significantly. In addition to the virus genome and polymerase, DNA building blocks (oligonucleotides) and specific search elements, so called primers, that are complimentary to the DNA region targeted, are needed. Buffer solutions and magnesium are also important to create an environment that is optimal for the polymerase to work in. In brief the PCR process includes the following steps:

1. Extraction of genetic material from the sample
2. Transformation of RNA to complimentary DNA (if the virus is an RNA virus), by the enzyme *reverse transcriptase*
3. Repeated amplification cycles;
    a. denaturation of dDNA to sDNA (temperature 95°C)
    b. hybridization / primer binding (temperature 58°C)
    c. elongation / polymerase copying (temperature 70-80°C)

Viruses are particularly apt for genome detection since, in spite of their small size, they all contain a complete set of genes. Even though the technique for PCR has been known for more than 25 years, it is in the last 10 years that the method has gained wide acceptance and use in diagnosing viral respiratory infections. The reasons for this are many, but some important factors are automated extraction, a shortened turnaround time at the laboratory, reduced costs, and the development of multiplex PCR methods, enabling the detection of several viruses in the same analysis.

Real-time PCR (also called quantitative real time PCR, qPCR) refers to a process by which the detection of nucleic acid can be measured continuously as the amplification cycles of the PCR method proceeds, instead of registering the detection after all 20-40 cycles. This can be achieved by adding a non-specific flourescent that binds to any double-stranded (ds) DNA (i.e. the PCR product), for example the *SYBR-green dye*. A disadvantage with the non-specific dye is that it binds to all dsDNA and therefore may also bind to unwanted sequences such as primer-dimers (a potential by-product of the PCR, consisting of primer molecules that have hybridized to each other, and are being amplified by the polymerase, leading

to competition for PCR reagents). A more specific real-time PCR method is achieved by adding specific oligonucleotide probes with a *reporter (or flourophore)* and *quencher* attached to the probe. The probe binds specifically to the sequence that is being analysed, and will not emit any fluorescence as long as the reporter and quencher are located in close proximity to each other. As the Taq-polymerase breaks the close proximity of the reporter and quencher on a probe that has bound it's target, a fluorescence signal can be detected, see figure 1 below.

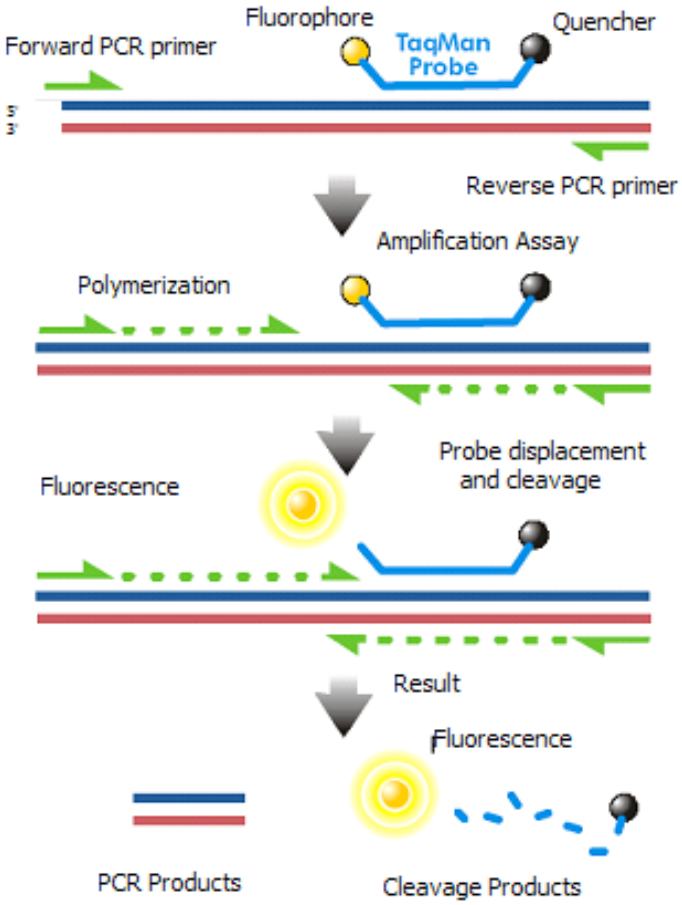

Figure 1. Schematic diagram of the TaqMan process in real-time PCR. Downloaded from open domain (http://en.wikipedia.org/wiki/File:Taqman.png)

Fluorescent signals can then be registered in each cycle of the PCR process. The cycle when this fluorescence becomes detectable is referred to as the cycle threshold value, or Ct-value, and is proportional to the logarithm of the target concentration before amplification.

Multiplex PCR refers to a process whereby several agents can be analysed in the same test run, by using multiple primer sets within a single PCR mixture. Amplicons (amplification products sized ≈ 80-150 bp) of varying sizes, specific to different DNA sequences, are produced. Careful optimization of annealing temperatures for each of the primer sets, and the optimal combination of primers are required. The main advantages of multiplexing are enhanced analysis capacity, shortened laboratory time and cost savings (less reagents needed). Multiplex PCR has facilitated rapid analysis of multiple respiratory agents in one test run, which in the case of ARTIs is important since one respiratory syndrome can be caused by several viruses or bacteria, making it difficult for the treating physician to choose which agent to look for. Multiplexing tends to hamper the performance of the amplification for many combinations, and therefore the reagent mixtures needs to be carefully evaluated.

Several new techniques, based on genome detection, are under development and all of these techniques are not accounted for in the thesis.

1.1.4 Serology

An infection with a certain organism can be diagnosed by measuring antibodies, produced by the host system as a response to infection by the agent. Serology still plays an important role in the screening of blood donors, diagnosis of viral hepatitis or HIV infections, and immune testing for a wide range of agents. The timing of specimen collection is important, since enough time need to elapse for the immune system to produce antibodies. Detection of an IgM response against the viral antigen suggests an acute infection, but often needs to be confirmed by a significant rise in IgG antibodies. Normally a fourfold rise in IgG is required, which means that at least two samples separated by 1-2 weeks or more is required. Serological tests are of limited use in immunocompromised patients who may not be able to mount a humoral response to infection.

Serology still plays an important role in the diagnosis of *Mycoplasma pneumoniae* and *Chlamydophilia pneumoniae*, even though the limitations of serology named above also apply to these agents. Genome detection, and in

particular real time PCR is an alternative to serology for diagnosis of *M. pneumoniae*, and it has been suggested that perhaps a combination of PCR and serology would be the optional method for diagnosing *M. pneumoniae* (38).

1.2 Respiratory viruses

The viruses described below are the viruses included in the PCR panel evaluated in this thesis. This list of respiratory agents is not intended as a complete list of all agents that cause acute respiratory tract infections, and some of the viruses described may cause symptoms outside of the respiratory tract. The description of each virus below focus on the respiratory illness they cause.

1.2.1 Rhinovirus

Human rhinoviruses (HRV) are small (30nm in diameter) single stranded non-enveloped RNA viruses, belonging to the *Enterovirus genus, family Picornaviridae* (which also includes enterovirus, parechovirus and hepatitis A virus), and were first isolated in 1956/-57 (39, 40). Rhinoviruses are highly heterogeneous genetically, and has since the discovery been classified into two distinct species, HRV-A with 74 known serotypes, and HRV-B with 25 known serotypes. Recently a new species of rhinovirus, HRV-C has been characterized (41-46). Most HRV serotypes have an optimal growth temperature of 33°C, the temperature of the nasal mucosa, and growth is generally restricted at temperatures above 37°C. Rhinovirus can survive on environmental surfaces at 24-37°C for hours to days (47) and in frosty conditions for years (48), but is inactivated in acidic conditions (PH< 6) and hence looses its infectivity in the human gastrointestinal tract. Because of HRV's lack of a lipid envelope, it is resistant to formulas such as ether, chloroform, fluorocarbon and detergents.

The HRV infection begins by delivery of the virus into the front of the nose, or the eye and then passes down the lacrimal duct, where infection of the epithelial cells of the posterior nasopharynx occurs. Introduction of HRV directly into the mouth or throat does not elicit infection efficiently (49). Transmission occurs both by person-to-person and by airborne transmission, but contaminated hands are probably the most important route of transmission (50). The incubation period is short, on average 2-4 days (median incubation period 1.9 days (51)), and has been reported in experimental studies to be as short as 8-12 hours (52, 53).

Yearly, human rhinovirus is believed to cause 30-50% of the cases of so called "common cold" (54, 55), but can amount to up to 80% of all upper respiratory tract infections during peak season (56), and mainly causes symptoms from the upper respiratory tract resulting in a mild disease. When HRV infect the upper respiratory tract it generally does not cause much damage to the epithelial cells it infects, and the symptoms induced by the

infection derives from the immune response rather than damage caused by the virus itself (57). However, complications such as acute otitis media (AOM) and acute community-acquired sinusitis (ACAS) are common (58). A causal role of HRV in AOM(59) and ACAS (60, 61) has been suggested. Recent studies have shown that HRV can in fact infect cells of the lower respiratory tract (62), and growing evidence further supports the association between HRV infection and development and aggravation of asthma (63-67). No antiviral treatment exists against HRV, so far.

1.2.2 Coronavirus

Human Coronaviruses (CoV) are large single stranded enveloped RNA viruses, belonging to the *Coronaviridae family*, and were first cultured in 1965 by Tyrell and Byone (68). The genome of CoV is the largest of all known RNA viruses. The prefix *Corona* denotes the crown-like appearance of the surface projections seen in electron microscope as described in 1967(69). Five human species of CoV, with a global distribution, have so far been described; the first one named CoV 229E in 1965-67, and shortly thereafter CoV OC43 was discovered (70). During the winter 2002-2003 a new and fatal disease, causing severe acute respiratory syndrome (SARS) in humans, emerged in the southern province of Guangdong in China and rapidly spread over the world. It was quickly identified as a coronavirus and subsequently named SARS CoV (18). After the SARS outbreak, which subsided by July 2003 after massive public health response, the increased interest for coronaviruses in general by the research community, resulted in the identification of an additional two human coronaviruses, CoV NL63 (19), and CoV HKU1 (20). Both these latter subtypes of CoV have since then been shown to cause disease in humans, display a worldwide distribution and phylogenetic analyses has proven them to be old viruses rather than new emerging viruses (71).

All non-SARS CoV replicate in the epithelial cells of the nasopharynx and after an incubation period of on average 3 days, cause mainly a relatively mild upper respiratory infection similar to that caused by HRV. It has been estimated that coronaviruses may account for up to 35% of all upper respiratory infections during peak viral activity, and that the overall proportion of adult colds caused by CoV is 15% (72). CoV affect all age groups but are more common in children, and reinfection occurs frequently. Co-infections with other respiratory viruses are common among CoVs. The different subtypes do not differ in their clinical presentation, apart from CoV

NL63 that seems to be more common in children with laryngotracheitis (croup) than other CoV (73, 74). The highest rates of infection are recorded in winter and early spring. Although most CoV infections cause a mild URTI there is evidence of more serious LRTIs as well, such as bronchiolitis and pneumonia in infants (75, 76), asthma exacerbation in children (77, 78) and adults (79), pneumonia in healthy adults (80), the elderly (81, 82), and in the immunocompromised host (83, 84).

SARS-CoV causes a distinctly different clinical picture compared with other CoV infections. Route of infection is most likely through the respiratory tract and although the focus of the disease is the lung, the initial clinical picture is that of a systemic spread with fever, myalgia, and laboratory findings such as leukopenia, pan-lymphopenia and thrombocytopenia. Cough and dyspnoea usually presents after a few days to a week (85), and severe respiratory symptoms including adult respiratory distress syndrome (ARDS) may develop late in the course of illness (86). The incubation period is significantly longer, 4-7 days (up to 10-14 days) than other non-CoV. No specific antiviral treatment is available for CoV infections, although several antiviral compounds, amongst them Ribavirin, were used during the SARS-CoV outbreak.

1.2.3 Adenovirus

Adenovirus belongs to the *Adenoviridae family,* and is a medium sized non-enveloped double stranded lytic DNA virus, that was first isolated from human adenoid tissue in 1953(87). As non-enveloped viruses adenovirus are highly resistant to physical and chemical agents, and can remain at room temperature for prolonged periods (up to 3 weeks), which gives them a high potential for spread. The virus is stable at low pH and resistant to gastric and biliary secretions, thus allowing replication in the gut (88). Adenovirus is the only DNA virus in the PCR assay evaluated in this thesis. So far, 52 human serotypes have been described.

Adenoviruses can cause a broad range of clinical syndromes, respiratory infection, epidemic conjunctivitis and gastroenteritis being the most common, and it is unclear why certain serotypes are associated with certain syndromes. Serotype 1-7,11, 12, 14, 16, 18, 21, 31, 34, 35 and 50 are associated with respiratory disease (72). Adenoviruses can persist as latent infections for years after an acute infection (89, 90). Transmission of respiratory adenovirus serotypes occurs through aerosolized droplets, but since the virus is often

secreted in faeces for a long time after an acute infection, the faecal-oral route probably constitute an important path for transmission. Adenoviruses have a worldwide distribution, and occur throughout the year. Most adenovirus infections are relatively mild in immunocompetent adults, but serotypes 3, 7, 14 and 21 have caused epidemics of severe respiratory disease in healthy adults (military recruits) (91, 92).

The respiratory infection, as it occurs in most cases, involves a mild pharyngitis and tracheitis, coupled with coryza. A syndrome of pharyngeal-tonsillitis with cervical adenopathy, which can be difficult do distinguish from group-A streptococcus infection, is associated with adenovirus (93). The infection can progress to a pneumonitis, especially in the immunocompromised host. Children are also prone to develop pneumonia caused by adenovirus. T-cell-mediated immunity is important for recovery after an acute infection, and patients who lack effective cellular immunity are at higher risk of adenovirus infection. Organ transplant patients and in particular hematopoietic stem cell transplant (HSCT) recipients can develop serious disseminated adenovirus infection (88). Currently there is no approved antiviral therapy for adenovirus infection. Cidofovir has good antiviral activity in vitro and has been reported to be of us in some animal models (94). Clinical experience of Cidofovir in humans rests on case reports and has been used mainly in paediatric and adult HSCT recipients with severe adenovirus infection, with varying results (95, 96).

1.2.4 Respiratory Syncytial Virus

Respiratory syncytial virus (RSV) is an enveloped RNA virus, belonging to the *paramyxoviridae family* that is divided in two main groups, type A and type B. It was first discovered in chimpanzees with coryza in 1956 (97), but was soon found to cause respiratory disease in humans (98). As the name implies the target cell of RSV infection is the epithelial cells of the respiratory tract. RSV infection does not induce immunity and repeated infections are common, although severe disease rarely occurs after the primary encounter. Transmission is through aerosolized droplets, and the incubation period is normally about one week (median 4.4 days (51)).

RSV infection is the most common cause of bronchiolitis among children, but also frequently causes pneumonia and tracheobronchitis (99-101). Acute otitis media is a common complication of RSV infection among children (102, 103). The infection typically affects infants within the first several

months of life, and every year the number of infants hospitalized because of pneumonia and bronchiolitis increase during the presence of RSV in the community, which occurs mainly in winter. RSV has been reported in several studies to display a biennial pattern, with a larger outbreak the first year, and the following year a somewhat smaller outbreak that starts later in the season compared to the previous year (104, 105). Although RSV infections are most frequent and cause the highest morbidity in infants, the infection can occur throughout life and recent attention has been given to RSV infection in the elderly. In patients older than 65 years the rate of hospitalization that was attributable to RSV in London was 0.7 per 1000, as compared to influenza A that had a rate of 1.1 per 1000 (106). Ribavirin, administered as aerosol, is approved for specific treatment of RSV infected infants with LRTI, but guidelines state that only for patients with severe disease or patients with high risk for severe disease, should be considered for treatment.

1.2.5 Metapneumovirus

Human metapneumovirus (HMPV) was discovered as late as 2001 (17), in nasopharyngeal secretions from 28 Dutch children with respiratory symptoms. The virus is a pleomorphic enveloped RNA virus that belongs to the *Paramyxoviridae family, subfamily pneumovirinae* (of which RSV is the most prominent member). Since 2001 it has been shown that HMPV is distributed worldwide and has been circulating for at least 50 years, undiscovered. Transmission is so far unknown but is likely similar to that of RSV (aerosolized droplets or direct contact with secretions), and reports on the incubation period are scarce but on average 4-6 days. Infections are most common in winter and are not seldom anti-cyclic with RSV (105).

Infections with HMPV resembles that of RSV, and are common in all age groups (107, 108) with symptoms ranging from mild URTI to bronchiolitis and pneumonia that may require mechanical ventilation. Peak incidence is during winter and occurs at a slightly higher age compared to RSV (109, 110). Fever, cough and coryza are the most common symptoms but the clinical picture is indistinct as with many RTIs. As a cause of LRTI in children HMPV is ranked second or third after RSV and IfA. HMPV infection can result in severe illness and pneumonitis, and even fatal disease in immunocompromised patients (111). No specific antiviral treatment is available for HMPV infections, but Ribavirin has been used in seriously ill patients (in vitro studies have shown equal antiviral activity against HMPV and RSV (112)).

1.2.6 Influenza virus

Influenza virus are large enveloped single stranded RNA viruses (80-120nm in diameter) that belong to the *Orthomyxoviridae family*, and are classified into three distinct types on the basis of antigenic differences, influenza A, B and C. Influenza A virus was first isolated from ferrets in 1933, influenza B in 1939 and influenza C in 1950. Influenza A virus is further divided into subtypes on the basis of their surface protein structures, hemagglutinin (H) and neuraminidase (N, or NA) activity, for example H1N1 or H3N2. The hemagglutinin is responsible for entry into host epithelial cells, and the neuraminidase is involved in the process of new virions budding out of host cells. Mutations in these surface proteins occur regularly, and by the random change of these surface proteins the virus is less likely to be recognized in an effective manner by the host immune system. When minor changes of the antigen H or NA take place, this is called an *antigenic drift,* which may cause more severe seasonal influenza outbreaks than normally occurs (antigenic drift can take place in all subtypes). In the case of *antigenic shift* (only IfA), which means that at least two strains of influenza combine to make up a new subtype with a mixture of surface protein from the original strains, pandemics can occur since no immunity exists in the community to this 'new virus'.

Influenza A causes epidemics yearly, and sometimes pandemics, whereas influenza B usually gives rise to local outbreaks. Recurrent epidemics of influenza have occurred for the last 400 years, and the most famous pandemic took place in 1918-1919 when three waves of influenza killed an estimated 21 million people worldwide.

The typical uncomplicated influenza commonly starts with acute onset of systemic symptoms like fever, chills, headache and myalgia. Incubation is 1-4 days (51), and systemic symptoms like fever usually persists for 3 days, but may last 4-8 days. Dry cough, pharyngeal pain and nasal congestion and discharge are also part of the illness and usually persist after the fever has subsided. The abrupt onset and predominance of systemic symptoms distinguishes influenza from several other respiratory viral infections. Influenza is associated with a U-shaped epidemic curve, with the highest attack rate among young people, whereas mortality is highest among older people with underlying cardiovascular and pulmonary conditions. Influenza C causes a mild disease and without the seasonality seen in IfA and IfB.

Influenza may give rise to primary viral pneumonia, mainly in patients with underlying disease, which begins with a typical onset but rapidly progresses to dyspnoea, cough and cyanosis, with bilateral infiltrates on chest X-ray

consistent with ARDS. During the recent 2009 H1N1 pandemic there were cases of previously healthy young individuals that suffered this complicated influenza infection. Another complicating manifestation of influenza can be a secondary bacterial pneumonia, which occurs after a period of improvement after the initial influenza infection (4-14 days), and typically *Streptococcus pneumoniae* or *Haemophilus influenzae* can be cultured. An increased frequency of *Staphylococcus aureus*, which is otherwise an uncommon cause of CAP, is seen in secondary bacterial pneumonia after influenza infection.

Antiviral therapy (Oseltamivir and Zanamivir) needs to be administered within 48 hours of start of symptoms, preferably within 24 hours, to have effect. Vaccines against influenza are available and are an important part of preventive measurements.

1.2.7 Parainfluenzavirus

Parainfluenzavirus (PIV) are large (150-200nm in diameter) enveloped RNA viruses belonging to the *Paramyxoviridae family.* PIV have been designated into five subtypes (type 1-3, 4a and 4b) that cause human disease, and PIV 1-3 are the most significant in humans. Parainfluenzavirus is a common cause of respiratory illness and their seasonal epidemiology depends on the type; PIV 1-2 has been reported to occur biennially usually during fall and early winter, PIV 3 is endemic throughout the year but with peaks in April-May and PIV 4 more irregularly and seldom (105, 113). PIV-3 are the most common type, as seen in studies of children (114).

Clinical manifestations are broad, but most result in an URTI, although a significant number (30-50%) are associated with AOM (115). About 15% of PIV infections causes LRTIs; PIV-1 being associated with croup, and PIV-2 and PIV-3 with bronchiolitis (114). As with many other respirator viruses PIV can cause severe disease in immunocompromised hosts, in particular HSCT and solid organ transplant patients. No specific antiviral therapy exists for PIV.

1.2.8 Enterovirus

Enteroviruses (EV) are small (30nm in diameter) non-enveloped RNA viruses that belong to the same *Picornaviridae family* as HRV. In contrast to HRV enterovirus replicate at a higher temperature (37°C) and is acid stabile

(pH 3-10), and can thus pass the gastrointestinal tract and give rise to a number of clinical syndromes. Poliovirus and Coxsackie virus A and B are examples of enteroviruses. Respiratory viruses are shed in secretions from the respiratory tract but also through faeces. Patients with enterovirus infection can shed virus two days before symptoms begin and up to 3 weeks after infection has subsided, therefore the incubation period is difficult to assess, and can range from 2 days to 2 weeks, but is usually 3-5 days. Distribution is worldwide. Infections occur endemically but in temperate climates peak in late summer and early fall.

Enteroviruses classically cause an unspecific febrile illness, but URTI and pharyngitis has been reported in 8-29% of children (116, 117), and throat examination is notable for only mild erythema without exudates or adenopathy. Fever is common. Specific syndromes are hand-foot-mouth syndrome (coxsackie A virus), herpangina (coxsackie A and B, EV -71), pleurodynia (Bornholm disease, caused mainly by coxsackie B virus) and fever/rash in young infants. Infection with EV rarely causes severe respiratory illness, but enterovirus 71 has been associated with outbreaks of hand-foot-and-mouth disease and herpangina accompanying severe multisystem disease, and even deaths (118). No specific antiviral treatment is approved for enterovirus infections, although there are case reports for the use of Pleconaril in treatment of severe coxsackievirus infection in children (119).

1.3 Atypical respiratory bacteria

The atypical respiratory bacteria that are discussed below were incorporated into the panel of agents included in the multiplex PCR since they often appear as a differential diagnosis of viral respiratory disease.

1.3.1 Mycoplasma pneumoniae

Mycoplasma pneumoniae was first described in 1944 by Eaton et al., and was initially known as the Eaton agent, and was believed to be a virus, since it passed through virus filters and did not respond to sulphonamides or penicillin. The organism was proposed the taxonomical designation *M. pneumoniae* in 1963 (120). *M. pneumoniae* is a short rod that has no cell wall (and hence does not respond to ß-lactam antibiotics) and is not visible on gram staining. There are several commensal mycoplasmal species, the most common being *Mycoplasma orale* and *M. salivarium* and these do generally

not cause disease in humans (case reports in immunocompromised patients are described) (121).

Clinical disease in the respiratory tract is characterized by an insidious onset of fever, malaise, headache and a dry cough. In 5-10 % the illness may progress to tracheobronchitis and pneumonia (72). In contrast to several respiratory viruses *M. pneumoniae* is not a common pathogen in patients with chronic obstructive lung disease (122). The incubation period is relatively long, 2-3 weeks (123), and the mode of transmission is person to person through cough, but relatively close association to the index patient seems to be required. Most cases of pneumonia are relatively mild, and certainly most cases of URTI resolve without the use of antibiotics,, but severe cases of pneumonia and even fatal cases do occur (124), most likely due to the organism's immunogenic properties.

The attack rate of infection is highest in the age group 5-20 years old, but can occur at any age. Children younger than 3 years of age primarily develop an URTI (125). Outbreaks of *M. pneumoniae* infection occur in closed populations like military recruit camps and boarding schools with a high rate of secondary infection (126). Treatment options include macrolides, tetracyclin or doxycyclin.

1.3.2 Chlamydophilia pneumoniae

Chlamydophilia pneumoniae is an obligate intracellular bacterial pathogen that infects epithelial cells of the respiratory tract, and can cause pneumonia. Estimates that *C. pneumoniae* cause about 5% of bronchitis and approximately 10% of CAP have been reported (127). Most respiratory infections by this organism are mild, and asymptomatic infections have been reported in both children and adults (128, 129). Co infection with other organisms, such as *Streptococcus pnuemoniae* and *M. pneumoniae* may occur frequently (130). The exact incubation period is uncertain but 21 days has been suggested. Onset of symptoms is like *M. pneumoniae* gradual. Treatment options are as with *M.pneumoniae*.

2 AIMS

The overall aim was to evaluate the use of a multiplex real time PCR assay for detection of viral respiratory infections, from a clinical perspective.

The specific aims were:

- To describe and validate the use of an in-house multiplex real-time PCR method for detection of respiratory viruses
- To examine the rate of detection, using the multiplex PCR assay, in adults with respiratory tract infections, in relation to the clinical presentation and the duration of symptoms in an outpatient setting
- To investigate, by means of a prospective study, if access to a method for rapid etiologic diagnosis of respiratory tract infections would have an impact on antibiotic prescription rates
- To describe the seasonal distribution of various respiratory pathogens, throughout the year with special reference to human rhinovirus infection, using the multiplex PCR assay

3 PATIENTS AND METHODS

Two prospective studies and two retrospective studies are included in this thesis. For a schematic overview see figure 2 below.

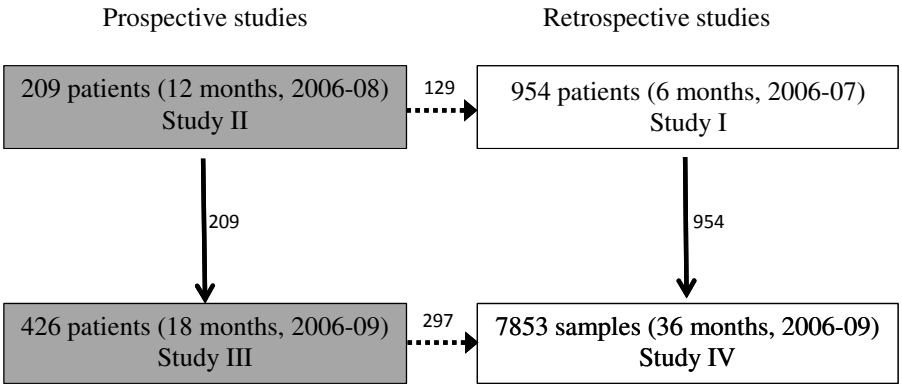

Figure 2. Schematic overview of studies included in this thesis.

3.1 Multiplex real time PCR assay

The multiplex real time PCR assay that is evaluated in this thesis was set up at the Department of Clinical Virology at Sahlgrenska University Hospital, Gothenburg, Sweden, during 2004-2005 and has, since November 2006 included the following respiratory agents;

- Rhinovirus
- Coronavirus (OC43, NL63, 229E)
- Enterovirus
- Influenza A virus
- Influenza B virus
- Parainfluenzavirus (1-3)
- Respiratory syncytial virus
- Metapneumovirus
- Adenovirus
- *Mycoplasma pneumoniae*
- *Chlamydophilia pneumoniae*

PCR assays are highly sensitive methods provided that the search elements, i.e. the specific primers, match the gene sequence of the virus or bacteria that is to be detected. In order to avoid mismatch because of mutations in the gene sequence of the agent, or because of heterogeneity of the agent, all primers and probes were designed to bind conserved segments of the target agents. This is particularly important for viruses with many subtypes, such as rhinovirus, enterovirus and adenovirus. The accuracy of the AdV-PCR has been documented by Heim et al. (131). The target region for the HRV/EV assays was the conserved segment of the 5' untranslated region that allows amplification of all subtypes, and which has been used previously by others (132-134). The primers and probes used for IfB and PIV 3 were developed by Dr Lars Nielsen, Copenhagen, those for PIV 1-2 have previously been described by Watzinger et al., those for CoV (NL63, 229E and OC43) by Gunson et al., and those for IfA was a modification of a system published by Ward et al.

The real-time PCR procedure evaluated, and used in all four studies, is based on automated specimen extraction and multiplex amplification (135) (Paper I). Nucleic acid from 100 µL of specimen was extracted into an elution volume of 100 µL by a Magnapure LC robot (Roche Molecular Systems, Mannheim, Germany), using the Total Nucleic Acid protocol, and was amplified in an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA). The amplification was carried out in 50 µL reaction volumes, including 10 µL of sample preparation and 25µL of one-step RT-PCR master mix from Applied Biosystems (this was exchanged to a master mix from Invitrogen (Carlsbad, CA) in 2007 to improve sensitivity). After a reverse transcription step at 46°C for 30 min followed by 10 min of denaturation at 95°C, 45 cycles of two-step PCR was performed (15 sec at 95°C, 60 sec at 58°C). Each sample was amplified in 5 parallel reactions, each containing primers and probes specific for 3 targets.

Pooling several agents into the same well in the PCR analysis, i.e. multiplexing, tends to hamper the performance of the amplification for many combinations. Therefore, the reagent mixtures were carefully evaluated and combinations were only accepted if the Ct-value in multiplex was no more than 2 cycles higher than when detection was carried out in separate reactions. The performance was evaluated using pUC57 plasmids with inserts of the targeted viral or bacterial sequences, synthesized by GenScript Corp. (Piscataway, NJ).
All samples tested for by PCR assay in study I-IV were analysed at the Department of Clinical Virology, Sahlgrenska University Hospital, Gothenburg, Sweden, and is referred to in the text as "the laboratory".

3.2 Paper I

954 samples from patients with symptoms of respiratory tract infection, which were referred to the laboratory during October 2006 through March 2007 (6 months), were analysed retrospectively, with the above-mentioned multiplex real time-PCR assay. Patients of all ages, both inpatients and outpatients were included. The specimens were obtained by swabs from the nasopharynx or oropharynx, and were jointly placed in a sterile container with 1 ml sodium-chloride solution and sent to the laboratory.

Analysis by multiplex real time PCR was carried out as described above (section 3.1).

3.3 Paper II

During two consecutive winter seasons (12 months), from October through April 2006-2008, 209 adult patients (≥18 years) with symptoms of an acute respiratory tract infection (ARTI), and duration of less than 2 weeks, were prospectively included. All patients visiting an outpatient clinic at either one of eight primary health centres or one of four infectious disease outpatient departments in Western Sweden (a region with a catchment area of approximately 600.000 inhabitants) were asked to participate in the study. An acute ARTI was defined as having at least two of the following symptoms; coryza, congestion, sneezing, sore throat, odynophagi, cough, chest pain, shortness of breath or fever, for which the physician found no other explanation.

Exclusion criteria were hospital-acquired infection (defined as > three days in hospital), duration of symptoms > 14 days, confirmed bacterial infection (defined as either a positive rapid group A streptococcus antigen test and physical findings consistent with a bacterial tonsillitis, a perforated AOM, a history and physical findings consistent with a lobar pneumococcal pneumonia, or a positive blood culture and history and physical findings consistent with septicaemia.

Signs and symptoms were recorded in a web-based case report form. Nasopharyngeal and oropharyngeal swab samples were jointly placed in a

sterile container with 1 ml of sodium-chloride solution and sent to the laboratory for analysis, with the above-mentioned multiplex real-time PCR assay. At the laboratory, specimens were either analysed directly or frozen at –70°C for later analysis. Ct values for each patient sample positive by real-time PCR were recorded for semi-quantitative estimation of the amount of DNA/RNA in each specimen. Patients were asked to return for a follow-up visit 10 days (+/– 2 days) after the initial visit. The same protocol was used at initial and follow-up visit.

A control group of 100 healthy adults without a history of fever or symptoms of ARTI during the preceding 14 days were also included. Control subjects were contacted for a telephone interview 2 days after testing and excluded if they had symptoms of ARTI, to avoid detecting virus that might be shed in high levels prior to onset of symptoms.

Analysis by multiplex real-time PCR was carried out as described above (section 3.1).

3.4 Paper III

The patients in study III were included during three consecutive winter seasons (18 months) during October through April 2006-2009, in the same setting as described in paper II. Patients included in study II constitute a subgroup of the study population in paper III (48%).

406 adult patients (≥18 years) with symptoms of an acute respiratory tract infection (ARTI) with duration of less than 2 weeks were prospectively included. All patients visiting either one of eight outpatient clinics or one of four Infectious Disease Outpatient Departments in Western Sweden were asked to participate in the study. An acute ARTI was defined as having at least two of the following symptoms; coryza, congestion, sneezing, sore throat, odynophagi, cough, chest pain, shortness of breath or fever, for which the physician found no other explanation.

Exclusion criteria were hospital-acquired infection (defined as > three days in hospital), duration of symptoms > 14 days, confirmed bacterial infection (defined as either a positive rapid group A streptococcus antigen test and physical findings consistent with a bacterial tonsillitis, a perforated AOM, a history and physical findings consistent with a lobar pneumococcal pneumonia, or a positive blood culture and history and physical findings

consistent with septicaemia. Patients with ongoing antibiotic treatment at the time of inclusion were excluded.

Patients were stratified according to duration of symptoms of either ≤5 days or >5 days. Open label randomisation was performed by means of a predefined randomisation list, using a central computer based procedure on the day of inclusion, with immediate result of randomisation given to the physician and patient on the day of inclusion. Randomisation resulted in the treating physician receiving the results of the multiplex real-time PCR analysis on the day following inclusion (rapid result) or 8-12 days later (delayed result). For a schematic outline of the study see figure 3.

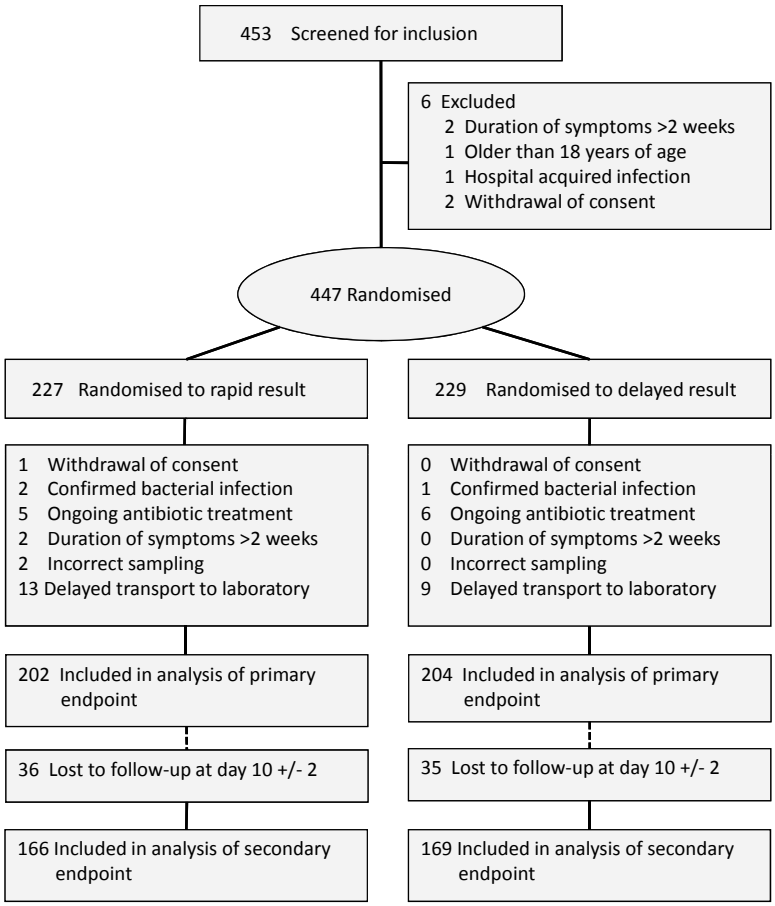

Figure 3. Study design paper III

Recruitment was performed Sunday through Thursday 8 am-5 pm, allowing for the laboratory to report rapid results within working hours the following day. Nasopharyngeal- and throat swab specimens were collected from each patient by swabs, which were jointly placed in a sterile container with 1 ml of sodium-chloride solution, and sent to the laboratory the same day. At the laboratory, specimens were either analyzed directly or frozen at –70ºC for delayed analysis.

Additional diagnostic testing, including throat and sputum cultures, CRP and X-ray investigations, as well as any treatment options, were left at the discretion of the treating physician and recorded in the case report form. Antibiotic prescription at initial visit was recorded and analysed in relation to access to a rapid vs. delayed result, and represents the primary endpoint of the study. Results in the rapid result group were provided to the treating physician within 24 hours in the majority of patients, and within 48 hours for all patients. Physicians were not instructed on how to act upon the given result of the PCR test. All patients were asked to return for a follow-up visit 10±2 days after the initial visit. The same web-based form was used at initial and follow-up visits. Antibiotic treatment (ongoing or initiated at follow-up visit) was recorded, and constitutes the secondary endpoint of the study.

Analysis by multiplex real time PCR was carried out as described above (section 3.1). Detection of a respiratory virus by PCR does not exclude concomitant bacterial infections or other non-infectious causes. This safety issue was discussed with the physicians at the beginning of the study and the investigators were encouraged to treat patients with bacterial complications at their own discretion.

3.5 Paper IV

Samples for this retrospective study were collected during November 2006 through October 2009 (36 consecutive months), and constituted nasopharyngeal samples only (n=8021). One sample per patient per day, both inpatient and outpatient samples from all ages were accepted for inclusion into the study, leaving 7853 samples from 7720 patients.

Analysis by multiplex real time PCR was carried out as described above (section 3.1)

In addition to the mulitiplex real-time PCR assay 703 samples were analysed with a single PCR assay (136) for detection of influenza A only, as a measure to meet the increased workload during the 2009 H1N1 influenza A pandemic.

Sequencing of selected rhinovirus positive samples was carried out to determine genogroup and perform phylogenetic analysis. Samples from late 2006 to early 2010 with a relatively high viral load (as defined by Ct-values <30) were chosen. A first round of amplification was carried out using primers Rhino_458F, CCGGCCCCTGAATGYGGCTAA and Rhino_1087R, TCWGGiARYTTCCAMCACCAiCC. If necessary, a second round of semi-nested PCR was run with Rhino_547F, ACCRACTACTTTGGG-TGTCCGTG and Rhino_1087R to obtain amplicons for sequencing. Cycle sequencing was then performed, using ddNTP (Big Dye, ABI) and the same primers as in PCR, followed by sequence analysis in an ABI 3130 XL automated sequence reader. The obtained sequences were aligned with database sequences representing all rhinovirus types, and phylogenetic comparison was performed by distance matrix analysis and neighbour-joining tree construction using the MEGA4 software (137).

3.6 Statistical analysis

The Chi squared test was used to compare proportions (paper II, III, IV), and a p-value of <0.05 was considered significant (paper II, III, IV). In paper II Spearman's rank correlation coefficient was used to analyse correlations.

In paper III, a prospective study with a primary endpoint to investigate antibiotic prescription in two randomized groups, a power calculation was done. The study was scheduled to include at least 200 patients in each group (rapid or delayed result), allowing for a statistical power of 80% to demonstrate an estimated reduction in antibiotic prescription rate from 20% to 10% in the rapid result group.

Multivariate analysis (paper III), using backward stepwise (Wald) logistic regression was carried out to analyse factors independently predicting a positive PCR results, or prescription of antibiotics.

Genetic distances (paper IV) were compared using Student T-test.

The SPSS software package, version 17.0 for Mac (SPSS Inc, Chicago, IL) was used for all statistical analyses (paper II, III, IV)

3.7 Ethical approval

The Regional Ethical Review Board in Gothenburg approved paper II and III, and all patients gave their informed consent. In paper I and IV all personal information was coded in a way that individuals could not be identified.

4 RESULTS AND DISCUSSION

4.1 Detection rates (paper I-IV)

The use of multiplex PCR assays for diagnosing respiratory viral infections has significantly increased the diagnostic yield and several previous studies have shown that the detection rate increases by approximately 50% compared to conventional diagnostic methods such as virus isolation, DFA and serology (31, 138, 139). The main reason for this is most likely that viruses like HRV, CoV and HMPV, which could not easily be detected by traditional methods, can now be detected (140).

In paper I, which included patients of all ages and samples collected during winter months, the positive yield was 48% (n=457/954), which is in line with previous studies displaying a detection rate ranging between 43%-63% (4, 31, 141, 142). Even higher rates have been reported in the young and very young children with short duration of symptoms (143, 144), reaching detection rates above 90%.

The higher detection rates recorded among children may have several underlying causes, such as:

- A higher incidence of respiratory viral infections in children compared to adults

- Greater viral exposure, for example children that are enrolled in day care have a higher consultant rate to primary care for ARTIs and higher prescription rate of antibiotics for ARTI, than children not attending day care (145, 146). In Sweden 83% of children aged 1-5 years are enrolled in day-care in 2003 (147)

- A prolonged viral shedding in children compared to adults has been reported for several respiratory viruses, like 2009-H1N1 IfA (148), Adv (149), and HRV (150).

- Lack of pre-existing immunity as well as an immature immune system may cause higher levels of viral replication, and prolonged viral shedding in children.

It is also possible that children tend to seek health care earlier in the course of disease than adults, and detection rate with real time PCR is inversely related to duration of symptoms, as described below (paper II).

Furthermore, in asymptomatic children, in contrast to asymptomatic adults (as was shown in paper II, see below), detection of respiratory viruses, in particular from the *picornaviridae family* is seen in relatively high frequencies (20%) (151). Rhinovirus and enterovirus found in asymptomatic children may represent a previous infection dating back 4-5 weeks, due to prolonged shedding of virus (150). Asymptomatic shedding of virus (HRV, RSV and PIV) has also been reported among immunocompromised patients (152, 153).

In paper IV, a retrospective study of patients of all ages (range 0-98 years, median age = 22 years), but with samples collected throughout three full calendar years, the detection rate was 44.5% (n=3496/7853). This detection rate is on the lower scale of reported positive samples with various NAATs. However, studies reporting higher detection rates comprised either data from younger patients and / or data collected during peak seasons for viral ARTIs. These are factors demonstrated in paper II and paper IV to increase the diagnostic yield. In an adult well defined population of patients with <2 weeks duration of symptoms (paper III), where physicians had excluded confirmed bacterial infections, the detection rate was 47% (n=191/406). Detection rates stratified according to age groups (paper IV) are depicted in figure 4 below.

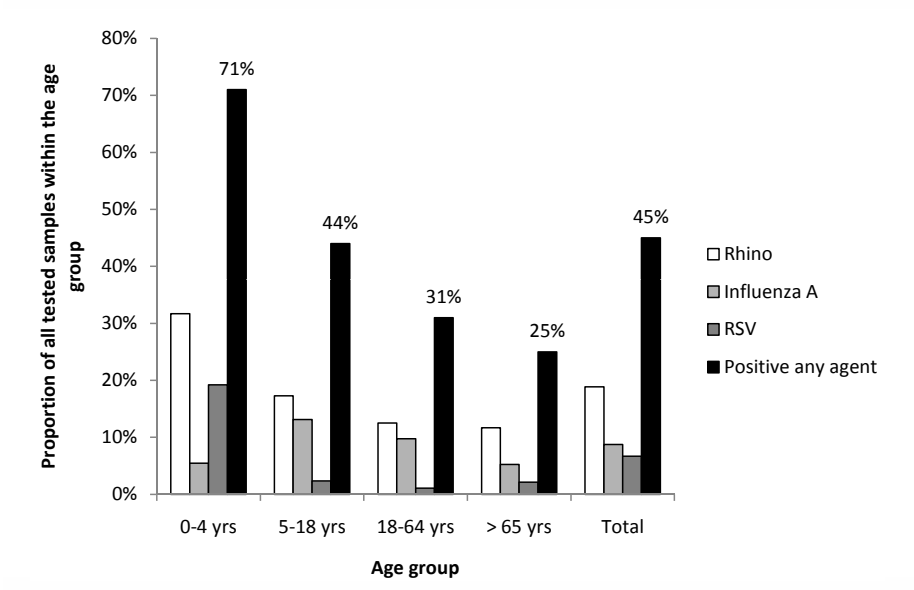

Figure 4. Proportion of samples positive by qPCR for any agent, rhinovirus, and influenza A virus, and respiratory syncytial virus (RSV) for all samples and stratified according to age groups (Paper IV). In manuscript

We included a control group (paper II) of 100 healthy subjects without symptoms of respiratory tract infections during the previous 2 weeks, and had follow-up two days after samples were collected, and found virus in only 2% of cases. Both of these were positive for HRV. Most studies do not include controls. One study of adults from primary care in the UK however found a respiratory virus, predominantly HRV, in 12% of controls that reported no signs of RTI during 2 months before sampling (4). However, that study did not follow up controls after sampling to rule out an early infection, which may explain their higher detection rate among controls in their study. Our result suggests that viral shedding exceeding 14 days in immunocompetent adults is rare.

In summary, detection rates of the PCR assay evaluated in paper I-IV (ranging between 43% and 47%) are comparable to previous studies of NAATs and reflect that genome detection yields a significantly higher detection rate compared to other conventional methods of etiologic diagnosis of respiratory viruses. A shortcoming of the PCR panel is that it does not include common bacteria that cause ARTI, like *Streptococcus pneumoniae*, *Streptococcus group A*, and *Haemophilus influenzae*. This might be of value, particularly in the diagnosis of adults with ARTI. Among children these bacteria frequently colonizes the oropharynx and nasopharynx without causing disease, making the interpretation of a positive result more complex. However, other tests apart from the PCR detection, like cultures from nasopharyngeal swabs, sputum cultures and / or urine-antigen tests for *Streptococcus pneumoniae* and *Legionella pneumoniae,* can be performed in addition to the multiplex PCR.

The notably high proportion of negative samples during summer and for patients older than 65 years of age raises new questions and warrants further investigation. Is this finding significant for a true lower prevalence of these agents among the elderly and / or during summer? Or are there other, so far unknown respiratory agents that cause respiratory symptoms in these patients?

The optimal sampling site for *M.pneumoniae* is not yet fully determined. Michelow et al. reported that nasopharyngeal and oropharyngeal samples were equally efficient in diagnosing *M.pneumoniae* with PCR, but that a combination of the two provided the best diagnostic yield (154). Another study showed that sputum was superior to nasopharyngeal / oropharyngeal samples (155). For most patients in routine care however, samples from the lower respiratory tract are not feasible, since few patients with *M. pneumoniae* have productive cough and invasive procedures are seldom

warranted, hence a pragmatic approach will likely include the use of NPH / throat swabs. A combination of PCR and serology is probably the optimal strategy for diagnosing these infections, as previously suggested by Waites (156), but adds complexity and costs to the procedure.

4.2 Duration of symptoms (paper II)

In paper II the impact that duration of symptoms may have on detection rate of the evaluated PCR assay, was investigated. The majority of patients (63%) were included within the first 7 days of disease. The detection rate was significantly higher in samples from patients with a duration of symptoms of 6 days or less (51%) than in samples from patients with a duration of symptoms of 7 days or more (30%, $p<0.01$), see fig 5 below. This finding is in line with the reports using PCR assays for influenza A that showed a correlation between detection rates and duration of symptoms (157).

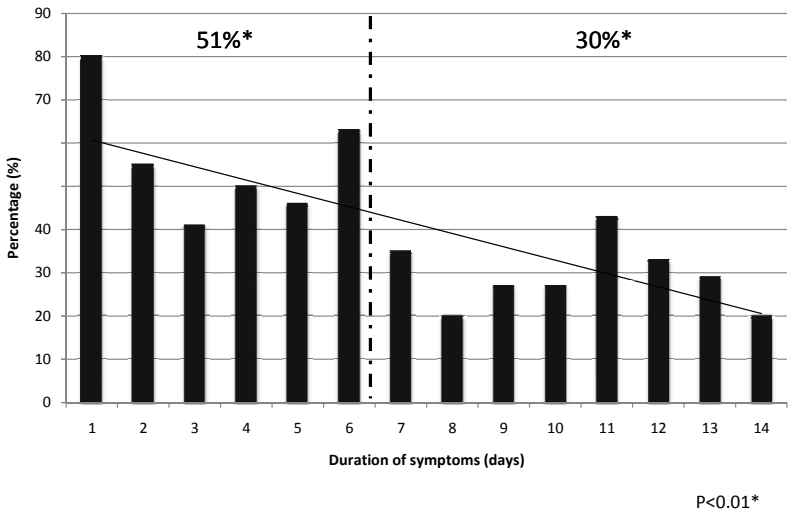

*Fig 5. Distribution of real-time PCR positive samples (n=94) according to duration of symptoms of patients (n=219). *51% vs. 30%, Chi-square test. Published in J Clin Vir 47 (2010) 263–267. (Paper II)*

Duration of symptoms should be taken into account when using these tests in clinical routine for diagnosing adults with ARTI's. Viral shedding may be longer in immunocompromised patients and young children (see above), and this correlation may not be applicable to those groups of patients.

4.3 Cycle threshold values (paper II)

Cycle threshold values, or Ct values reflect the relative amount of virus RNA/DNA found in the sample analysed. A low Ct value corresponds to a relatively high amount of virus and vice versa. However, the semi-quantitative estimation of viral loads by Ct values must be interpreted with caution since the amplification efficiencies were not identical for the different component PCRs, and quantification standard were not included. To some extent, the probability that a given agent constitutes an etiological agent should be related to the Ct value: The lower Ct value, the higher the probability, and this may also be of relevance for the interpretation of samples where > 1 agent is detected, especially in viruses with relatively low variability.

For several viruses analysed in paper II, the amount of viral DNA/RNA decreased with duration of symptoms, suggesting a gradual reduction of viral shedding from the epithelial surface over time. This is in concordance with the gradual reduction of RSV levels in nasopharyngeal aspirates described by Gerna et al. (158) and Campanini et al. (159).
We found a correlation between Ct values and duration of symptoms for CoV, IfA, and IfB ($n = 13$, $rs = 0.33$, $p < 0.05$; $n = 24$, $rs = 0.17$, $p < 0.05$; $n = 10$, $rs = 0.65$, $p < 0.01$), but not for HRV, as is shown in figure 6 below.

The high genetic variability of HRVs increases the probability of mismatches between target and primer or probe. Such mismatches may result in a higher Ct value and explain the lack of correlation between duration of symptoms and Ct values for HRV. For other viruses, such as influenza B virus, with less genetic variability viral detection will be more stable. The lack of association between duration of illness and Ct values in HRV infection may also reflect variations in viral shedding and differences in pre-existing immunity between individuals as well as limited sample size.

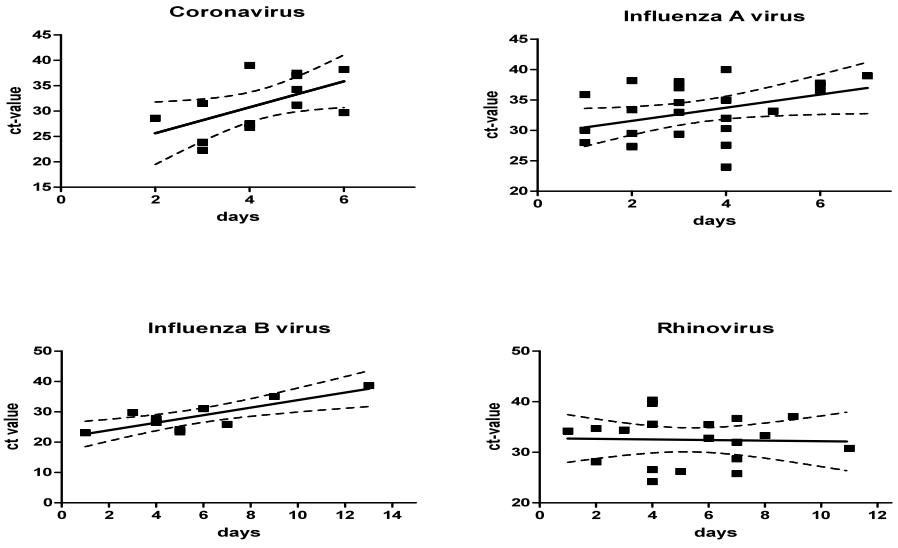

Figure 6. Cycle threshold values (Ct-values) in relation to duration of symptoms in patients with acute respiratory tract infections. Human Corona virus OC43 and NL63, influenza A virus, influenza B virus and rhinovirus. Published in J Clin Vir 47 (2010) 263–267. (Paper II).

4.4 Follow-up testing (paper II)

In paper II 63% of patients (n=138) came back for a follow-up visit 10+/– 2 days after initial visit. 57 patients with a positive sample at initial visit were sampled with a follow-up PCR test. 13 patients (23%) were positive for the same agent at initial visit and at follow-up visit, of which 8 patients (63%) were positive for HRV, see table 1.

Table 1. Follow-up (10+/-2days after initial visit) test result from analysis with qPCR of nasopharyngeal /throat swab specimens. Published in J Clin Vir *47 (2010) 263–267. (Paper II)*

Patients and Samples	N (%)
Patients at follow-up visit	138 (63)
Patients with positive PCR analysis at initial visit	94 (43)
NPH[1] and throat swab samples collected at follow up	57 (61)
Patients with positive PCR analysis at initial and follow-up visit	18 (32)
Patients with same pathogen at initial and follow-up visit	13 (23)
Pathogens	
Rhinovirus	8 (62)
Human Coronavirus NL63	2 (15)
Human Coronavirus OC43	1 (8)
Mycoplasma pneumoniae	1 (8)
Respiratory Syncytial virus	1 (8)

[1] NPH = Nasopharyngeal

All patients still positive for the same agent on follow-up had higher Ct-values 10 days later (data not shown). Patients positive for HRV at both initial visit and at follow-up visit, and their Ct values are depicted in figure 7. All patients except for one had a higher Ct value at follow-up visit.

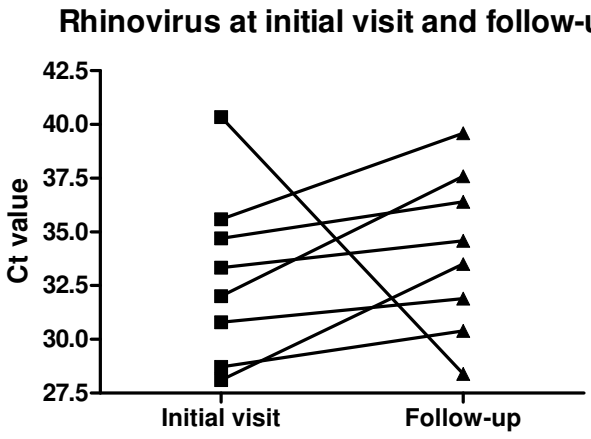

Figure 7. Ct-values of 8 patients positive for rhinovirus at initial visit and at follow-up visit. Unpublished data

The fact that relatively few patients were still positive for the same virus at follow-up visit, and that all but one patient had lower amounts of virus (as estimated by Ct values), suggests that adults with viral respiratory tract infections in the majority of cases clear their infection within two weeks, and that viral replication stops. HRV could possibly be an exception to this pattern with a prolonged viral shedding, as has been described among children (150).

The viral decline rate can be estimated by comparing the Ct value on the second and first occasion. For rhinovirus the Ct values increased by a median of 2 cycles after 10 days (as seen in figure 7), suggesting that the rhinovirus concentration had decreased to 50 %. For the other viruses this calculation was not possible, because the virus was rarely detected in the second sample. However, for influenza B virus there was a good correlation between symptom duration and Ct value. The coefficient of the regression line for this correlation should be a reflection of the viral decline. This coefficient was 2.5 cycles per days, indicating that the influenza B virus concentration would decrease by 6-7 logs after 10 days (assuming that one log corresponds to 3.5-4 cycles, which is in accordance with our evaluations of the PCR efficiency in the multiplex PCR). This indicates that the decline rate for IfB virus may be as much as 10 times faster than for HRV. The rapid decline of IfB virus agrees with the fact that it was never detected in the follow-up samples. The very slow decline of HRV suggests that patients shed HRV for a longer period of time compared to other viruses examined. Whether this translates into longer period of transmission as well as infectivity remains to be determined.

4.5 Symptoms and physical findings (paper III)

In the prospective study symptoms were recorded in a standardised web-form for each patient at both initial visit and at follow up visit. No pattern of specific symptoms was found that could guide the physician to an etiologic diagnosis. Nasal congestion / coryza was the most frequently reported symptom (n=338, 83%), followed by a sore throat (n=310, 76%) and a reported headache (n=298, 74%). Fever was reported in about half of the patients (n=215, 53%). In multivariate analysis, fever was independently predictive of a positive PCR result, and a sore throat was significantly more common among patients with a negative PCR result. Vomiting was the only symptom that was significantly more common among patients receiving an

antibiotic prescription at initial visit. One possible explanation of this could be that the treating physician interpreted vomiting, in this cohort of patients, as a sign of serious illness, and therefore were reluctant to withhold antibiotics. All patients that were positive for a microbial agent at follow-up visit displayed symptoms of an ARTI, i.e. no asymptomatic patients were discovered at follow–up visit. This further supports the notion that adult patients with symptoms of ARTI, and without obvious immunosuppression, rarely are asymptomatic carriers of virus in their upper respiratory tract.

Analysis of C-reactive protein (CRP) is commonly used by physicians to aid in the differentiation between viral and bacterial infection of the respiratory tract (160). The use of CRP in diagnosing LRTI however, has been questioned in a relatively recent meta-analysis (161). The systematic use of Procalcitonin, another biomarker of bacterial infection, has been reported to reduce antibiotics in hospitalized patients with CAP (162, 163), and may be an interesting way forward in antibiotic stewardship in the future. In an outpatient setting Procalcitonin did not however prove superior to CRP in diagnosing CAP in one study (164). Procalcitonin further lacks the additional information that is gained by an etiologic diagnosis, such as information about possible complications, the prognosis, and the risk of further spread of disease. The procalcitonin test has not been evaluated in our studies and is not further discussed here.

In paper III CRP was analyzed in 301 patients of 406 (74%), of which 39 patients (13%) had a CRP ≥50mg/L and 15 patients (5%) had a CRP ≥100mg/L. We found similar detection rates of the multiplex PCR among patients with a CRP level less than 50mg/L, higher than 50mg/L, and as in the overall study-population (n=127 of 262, 48%, n= 24 of 39, 62%, n= 191 of 406, 47% respectively). Hence a cut-off value of 50mg/L according to this study is not sufficient to differentiate viral form bacterial infection. In patients with a CRP level higher than 50mg/L a virus was detected in 46%, and in patients with a CRP higher than 100mg/L (n=15) four patients were positive for a virus and four patients were positive for a bacteria (*M. pnumoniae*). These figures indicate that although the patient may have at least moderately elevated CRP levels, the infecting agent may well be a virus. However, some caution must be applied in regards to the interpretation of CRP levels as a sign of bacterial or viral infection, since concomitant bacterial infections not tested for in this study are possible. Government guidelines for management of LRTI in Sweden (165) do not recommend the wide use of CRP as a diagnostic tool in cases where the suspicion of pneumonia or acute bronchitis is already high, but recommends the use of CRP for cases of uncertain LRTI, and suggests an interpretation of the CRP result as follows;

- CRP > 100mg/L: pneumonia likely – consider antibiotics
- CRP > 50mg/L + fever/dyspnoea/cough more than 1 week: pneumonia likely – consider antibiotics
- CRP < 20mg/L: pneumonia not likely – no antibiotics.

Antibiotic prescription rates have been shown to increase with rising CRP levels (166). This was true also in our study. Of the 39 patients with a CRP level ≥ 50mg/L, 12 (31%) received antibiotic treatment at initial visit, compared to 19 of 262 patients (7%) in the group of patients with a CRP level < 50 mg/L ($p < 0.0001$).

Chest x-ray was performed on 40 patients, and 10 of these had radiographic signs of pneumonia. The PCR results in the group of patients with infiltrates on chest x-ray were as follows: M. pneumoniae (n=3), HRV (n=1), CoV (n=1), HMPV (n=1) and negative result (n= 4).

4.6 Effect on antibiotic prescriptions (paper III)

The overall prescription rate of 8.4% in paper III was low compared to previous studies of ARTI in primary care (2). A recent thesis on treatment of RTIs and AOM in Sweden (167), reported an antibiotic prescription rate for RTIs in general (including AOM) of 45%, acute bronchitis; 60% and for the common cold antibiotics were prescribed in 16% of visits to the primary health care doctor. Although an overall reduction in the use of antibiotics for RTIs in Sweden has been reported (between 1999-2005 a reduction of 33% has been noted), as well as in other countries such as the United Kingdom (168), our finding is remarkably low. This could partly be due to a bias in our study population with a possible shift towards less severe infections. Another explanation could be that the treating physicians to a greater extent than usual followed the national guidelines for the treatment of uncomplicated ARTIs, or possibly that physicians improved their diagnostic skills during the studyperiod, considering that they received feedback regarding the underlying infectious agents as the study progressed.

In the group of patients that were randomised to receive a rapid PCR result the prescription of antibiotics at initial visit were reduced by more than half compared to the group randomised for a delayed result, see table 2. This finding suggests that access to a rapid and broad etiologic diagnosis of ARTIs

in an outpatient setting reduces antibiotic prescription rates at initial visit. Our study evaluated the impact of having access to a test rather than the impact of the actual result of the test. This finding implies that the mere prospect of a rapid etiologic diagnosis can influence the therapeutic decision in patients with an indistinct clinical presentation. The effect of having access to the test was even more pronounced in the group of patients with a CRP level > 50mg/L, with 11 out of 18 patients received antibiotic treatment in the delayed group. These results are in line with the observed reduction of antibiotic prescriptions when rapid diagnostic tests for influenza A virus was used systematically for hospitalized adults (169) and for children (170).

Table 2. Number of patients (n,%) that were prescribed an antibiotic at initial visit for acute respiratory tract infection and type of antibiotic, stratified according to randomisation to rapid (within 24 to 48 hours) or delayed (after 8-12 days) multiplex PCR analysis result. *Chi-square

Antibiotic prescription	**Rapid analysis (n=207)**	**Delayed analysis (n= 219)**	**P**
Initial antibiotic treatment, n (%)	9 (4.5)	25 (11.7)	0.005*
β-Lactam[a], n (%)	4 (2.0)	13 (6.1)	-
Tetracycline, n (%)	4 (2.0)	8 (3.8)	-
Macrolide, n (%)	1 (0.5)	3 (1.4)	-
Quinolone, n (%)	-	1 (0.5)	-

[a] Phenoxymethylpenicillin or Amoxicillin +/- Clavulanic acid or Loracarbef

A total of 335 (83%) patients (paper III) returned for a follow-up visit or were available for telephone appointment (visit n=243, telephone n=92), 166 of 202 patients (82%) in the rapid result group and 169 of 204 patients (83%) in the delayed result group. A group of 28 patients out of 406 (6.9%) who were not treated with antibiotics at first visit, reported ongoing antibiotic treatment at the follow-up visit, 19 patients out of 202 (9.4%) in the rapid result group and 9 patients out of 204 (4.4%) in the delayed result group. Hence, there was no significant difference in antibiotic prescription rates between the two groups at the follow-up visit. However, since some patients were lost during follow-up, the selection of patients may be biased. Moreover, physicians outside the study prescribed antibiotics to some patients in between initial visit and follow-up visit. Therefore these results from the follow-up visit in paper III should be interpreted with caution. In the absence of an algorithm on how to act upon the result of PCR testing, including a predefined antibiotic management plan, the observed reduction of antibiotic use at initial visit may be lost.

Patients that were positive for *M. Pneumoniae* or *C. Pneumoniae* (n=8) received an effective antibiotic treatment based on clinical assessment alone in only one quarter of the cases, supporting the need for reliable diagnostic tools for etiologic diagnosis of ARTIs. The fact that 41% of the patients who received an antibiotic at initial visit were positive for a respiratory virus also supports this notion.

Distinguishing viral from bacterial aetiology in ARTIs is often difficult on clinical grounds alone and vital signs, CRP and X-ray findings have a low predictive value (171, 172). The positive predictive value (PPV) of doctors in primary care diagnosing pneumonia, on patient history and physical examination, ranges between 19-24% (172-174). This may lead to over-prescription of antibiotics for infections misinterpreted to be caused by bacteria rather than viruses. However, Lieberman et al reported that physicians in primary care could exclude pneumonia in febrile adults with LRTI, based on patient history and physical examination, with a high degree of certainty (negative predictive value, 97%), but that the PPV was only 27%. It has been demonstrated that a fictitious patient history may mislead the physician in the examination of healthy individuals, causing over-prescription of antibiotics (175-177).

Reducing unnecessary use of antibiotics, and providing the patients with ARTI that really need them with adequate antibiotics, is of utmost importance and cannot be accomplished by a single strategy. Patient and physician oriented educational programmes could play an important role, as has been previously suggested (178). The use of biomarkers such as PCT may also contribute to this, but more studies are needed in the primary care setting before this can be realised. However, these programmes do not solve the issue of lack of an etiologic diagnosis, which is important not only for antibiotic stewardship, but also for addressing issues such as possible complications, prognosis, antiviral treatment, surveillance and infection control.

The findings in paper III support the implementation of similar multiplex PCR methods in routine clinical care, in order to reduce over-prescription of antibiotics in ARTIs. Ideally, an algorithm that can support the treating physician in how to act on a given PCR result, with an antibiotic / antiviral management plan included, should be in place. Since introducing such a diagnostic tool may have major ramifications on clinical care, further large clinical trials that include follow-up data on antibiotic prescription, as well as evaluation of health economics are needed to address this issue.

4.7 Microbial findings (paper III, IV)

In the prospective study of adults (paper III), with samples collected during winter months, influenza A was the most frequently detected agent (28%), followed by rhinoviruses and coronaviruses (22% and 16% respectively), see table 3.

Table 3 Positive results (multiple infections not included) of multiplex real-time PCR analysis of all included patients (in order of frequency), and positive results in Rapid vs. Delayed result group. (Paper III).

Detected Pathogens	**All patients included, n (%)**	**Rapid result group, n (%)**	**Delayed result group, n (%)**
Influenza A virus	56 (29.3)	31 (32.0)	25 (26.6)
Rhinovus	40 (20.9)	24 (24.7)	16 (17.0)
Coronavirus (all subtypes)	29 (15.2)	11 (11.3)	18 (19.1)
Coronavirus OC43	16 (8.4)	4 (4.1)	12 (12.8)
Coronavirus NL63	11 (5.8)	5 (5.2)	6 (6.4)
Coronavirus 229E	2 (1.0)	2 (2.1)	-
Respiratory Syncytial virus	18 (9.4)	6 (6.2)	12 (12.8)
Influenza B virus	14 (7.3)	7 (7.2)	7 (7.4)
Metapneumovirus	14 (7.3)	6 (6.2)	8 (8.5)
Parainfluenzavirus 1-3	7 (3.7)	4 (4.1)	3 (3.2)
Mycoplasma pneumoniae	7 (3.7)	5 (5.2)	2 (2.1)
Adenovirus	4 (2.1)	2 (2.1)	2 (2.1)
Enterovirus	1 (0.5)	-	1 (1.1)
Chlamydophilia pneumoniae	1 (0.5)	1 (1.0)	-
Total	191 (100)	97 (100)	94 (100)

In paper IV rhinovirus was the most commonly found virus (n=1136, 32,5% of positive samples), followed by RSV (n=411, 11,8%) and seasonal IfA (n= 345, 9,9%), see table 4 (following page).

Table 4. Results (n) of 7853 nasopharyngeal samples analyzed by multiple real-time PCR in relation to month of the year during the study period (Nov 2006- Oct 2009).
Positive results displayed in order of frequency. Proportion of positive results in relation to total amount of samples and in relation to total amount of positive samples (%). (paper IV, unpublished)

		Month														
		Jan	**Feb**	**Mar**	**Apr**	**May**	**Jun**	**Jul**	**Aug**	**Sep**	**Oct**	**Nov**	**Dec**	**Total n**	**Total %**	**% of positive**
Agent	Rhinovirus	60	75	70	75	99	96	71	80	194	180	75	61	1136	14,5	32,5
	Respiratory Syncytial virus	95	107	77	28	10	7	1	1	0	2	14	69	411	5,2	11,8
	Seasonal influenza A virus	133	82	68	6	4	3	0	0	0	4	5	40	345	4,4	9,9
	2009 H1N1 virus	0	0	0	0	3	16	86	36	31	107	0	0	279	3,6	8,0
	Adenovirus	15	19	19	7	17	9	9	13	7	21	19	27	182	2,3	5,2
	Parainfluenzavirus 1-3	8	10	18	23	19	20	9	3	4	25	18	17	174	2,2	5,0
	Metapneumovirus	37	24	12	11	3	1	1	1	1	2	4	25	122	1,6	3,5
	Influenza B virus	4	17	28	27	8	7	0	0	2	0	3	0	96	1,2	2,7
	Mycoplasma pneumoniae	15	6	3	2	4	2	4	6	4	20	10	16	92	1,2	2,6
	Coronavirus OC43	20	14	6	3	2	2	0	3	2	9	5	16	82	1,0	2,3
	Coronavirus NL63	5	22	11	2	6	1	0	0	0	0	0	0	47	0,6	1,3
	Coronavirus 229E	4	4	2	1	3	7	1	1	0	0	0	1	24	0,3	0,7
	Enterovirus	0	0	1	1	0	4	1	3	1	6	4	1	22	0,3	0,6
	Chlamydophilia pneumoniae	0	1	1	1	0	0	1	0	1	1	1	0	7	0,09	0,2
Type	Two agents detected	59	68	45	32	33	24	23	17	22	44	23	57	447	5,7	12,8
	Three agents detected	6	3	6	1	2	3	0	0	2	2	0	5	30	0,4	0,9
	Negative	348	277	244	177	189	191	482	549	927	546	219	208	4357	55,5	-
	Total	809	729	611	397	402	393	689	713	1198	969	400	543	7853	100	100

During the 2009 H1N1 pandemic, and within the study period (in Sweden June-Oct 2009), a remarkable increase in the number of samples referred to the laboratory for viral respiratory testing occurred. 3213 NPH samples were analysed, of which 703 (22%) were analysed targeting influenza A only. The 2009 H1N1 virus was found in 279 of 3213 samples (8.7%), 69 of 703 samples (9.8.%) with the single PCR, and in 210 of 1551 samples (13.5%) with the multiplex PCR. The testing during this period resulted in a considerable proportion of negative samples and thus the diagnostic yield decreased. Since a substantial proportion of samples were analysed for influenza A only it is possible that the occurrence of other viruses during this time period has been underestimated.

Multiple agents were detected among adults (paper III) in 12 patients (6.3% of positive samples), and in the larger retrospective study that included all ages (paper IV) in 477 of 3496 patients (13.6% of positive samples). HRV was the most frequently detected virus (n= 346, 72.5%) in samples where two or more agents were detected. Previous studies have reported a dual infection rate ranging between 7-20%, with a higher rate among children (105, 141). The clinical impact of multiple detections is controversial and remains to be determined. In one study of children with RSV infection, higher fever, longer hospital stay and more frequent use of antibiotics were associated with detecting several viruses in the same sample (179). Some evidence points towards a more severe disease in infants with bronchiolitis and co-infections with RSV and HRV, or RSV and HMPV (180-182). The use of Ct-values for a relative estimation of the amount of viral DNA / RNA may be of use when determining the clinical relevance of a positive test, particularly in samples where more than 1 virus is detected.

4.7.1 Age distribution (paper IV)

Positive yield was highest in young individuals and decreased with age of the patient for HRV, IfA and for RSV, as well as for any agent (see figure 4 above under detection rates, 4.1). A significantly higher positive yield was found among patients < 18 years old (61.5%, n=2187 of 3558 samples) compared with adults (30%, n=1309 of 4295 samples, $p<0.001$). The rather low detection rate in patients older than 65 years (25%) warrants further investigation, but reasons for testing in this age group may be different compared with children and young adults. The differential diagnostics against other respiratory conditions than infections may be important and the

informative value of a negative test result should not be underestimated in patients older than 65 years of age.

4.7.2 Seasonal distribution (paper IV)

In paper IV samples positive for any agent were significantly more common during winter and early spring (October through April, n=2439 of 4458 samples, 54,7%) compared with summer (May through September, n=1057 of 3395 samples, 31.1 %, p<0,001). If specimens that were analysed for IfA during the 2009 H1N1 pandemic (n=1662, 52%) were excluded, the positive yield was still significantly higher during winter (n=2267 of 4048 samples, 56.0%) compared with summer months (n=717 of 1556 samples, 45.8%, p<0.001). Stratified according to age the positive yield for children was 68.3% (n=1658 of 2428 samples) during winter, and 46.8% (n=529 of 1130 samples) during summer. For adults the rate of positive samples during winter was 38.5% (n=781 of 2030 samples) and during summer 23.3% (n=528 of 2265 samples, p<0001).

HRV was detected in a high proportion of positive samples during the summer months, as depicted in figure 8.

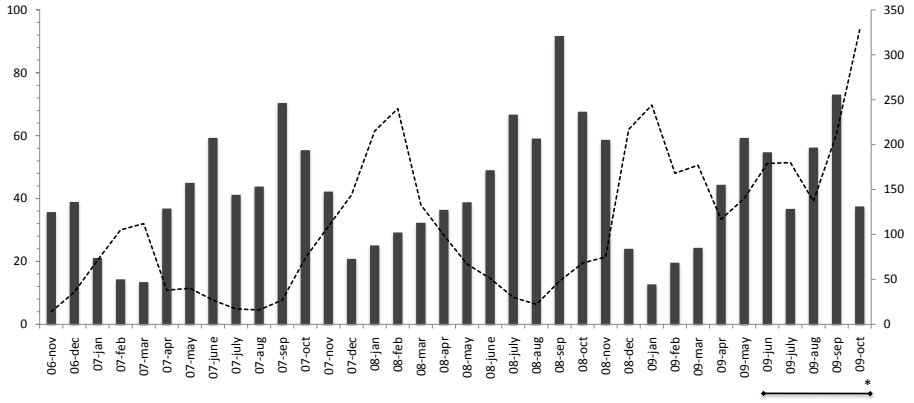

*Figure 8. Number of samples positive by qPCR for human rhinovirus (HRV) as proportion of total amount of positive samples each month, from November 2006 through October 2009. Total number of samples analysed depicted on right y-axis (dotted line). * During Jun 2009-Nov 2009 703 samples of 3213 (22%) were analyzed by single PCR for influenza A virus.*

IfA occurred with distinct peaks in midwinter (Jan-Mar) and not during summer (July-Sep). The 2009 H1N1 virus differed from this pattern with the start of the pandemic in Sweden in May 2009. RSV displayed a similar pattern to IfA with peaks in midwinter and a total absence of positive samples during summer months. RSV further showed a 2-year pattern with alternating early onset (peak in Jan 2008) and late onset (peak in Mar 2007 and 2009). HRV was present all year around, with the highest proportion of positive samples during September of each year, see figure 9 and 10 (following pages)

Several respiratory viruses have been shown to display a typical seasonal distribution in temperate climates, hence the traditional term "common cold". The best-described examples are RSV, IfA and to some extent HMPV, which usually peak in mid-winter (183-185), but seasonal occurrence of IfA and RSV has also been demonstrated in tropical climates (186, 187). PIV 1-2, RSV and HMPV have all been reported to display a 2-year rhythm (104, 105, 188).

It has been suggested, in observational studies, that the seasonal incidence of many respiratory viruses may be influenced by weather conditions. An inverse relation between the incidence of RSV infections and temperature, as well as between HRV infections and increased humidity has been reported (183, 189). Whether this has to do with the outdoor climate *per se* or other confounding factors such as for example indoor crowding during the cold season, is not fully understood. Others have argued for a possible interference between different viruses as a contributing factor to the seasonal distribution of respiratory viruses (105, 190, 191). For example, it has been suggested that HRV infections could reduce subsequent RSV and IfA infections, by inducing a strong interferon response, thereby creating a non-favourable environment for these viruses (192-194).

Positive samples for HRV occurred independently of other common viruses such as IfA and RSV throughout the study period. In absolute numbers HRV infections peaked during the severe RSV season, along with IfA (Jan-Feb 2008), and during the 2009 H1N1 influenza A pandemic (Sep-Oct 2009), although there was a bias towards increased testing during those time periods. Thus, it seems possible that different viruses have synchronous peaks, independent of each other. Interestingly, the proportion of all positive samples that were positive for HRV was highest during the summer months (May-Sep) during all three study-years, with an average detection rate for HRV of all positive samples of more than 50% (52%, 61% and 56% respectively).

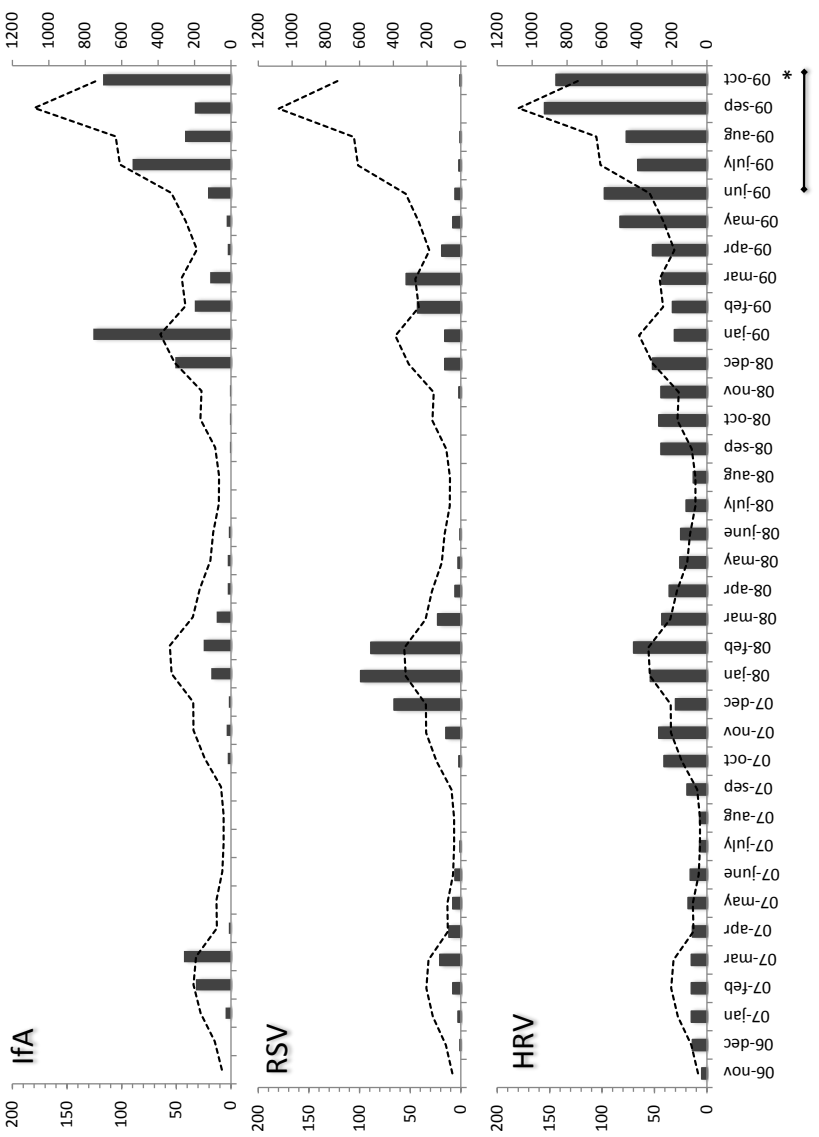

*Figure 9: Number of samples positive for influenza A (IfA), respiratory syncytial virus (RSV) and human rhinovirus (HRV) by multiplex real-time PCR each month from November 2006 through October 2009. Total number of samples analysed depicted on right y-axis (dotted line). * During Jun 2009-Nov 2009 703 samples of 3213 (22%) were analyzed by single PCR for influenza A virus.*

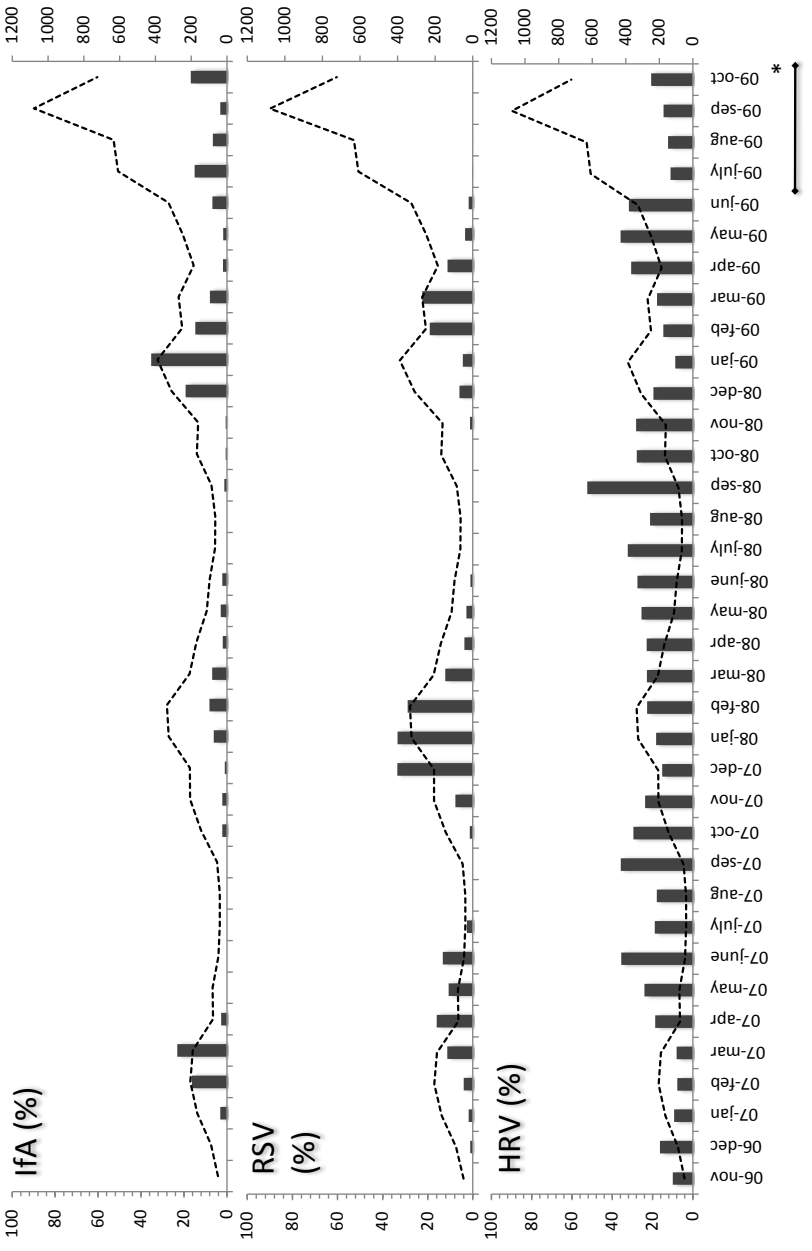

*Figure 10: Proportion of total number of samples analyzed positive by multiplex real-time PCR every month, for influenza A (IfA), respiratory syncytial virus (RSV) and human rhinovirus (HRV), from November 2006 through October 2009. Total number of samples analysed depicted on right y-axis (dotted line). * During Jun 2009-Nov 2009 703 samples of 3213 (22%) were analyzed by single PCR for influenza A virus.*

September displayed the highest proportion positive HRV samples in all three seasons, ranging between 70-92% of the positive samples. The seasonal peak for HRV in early fall and spring was described in studies based of viral isolation already in the 1960's (195), and has been confirmed in recent studies using NAATs (196). The overall seasonal pattern of HRV infections in our study however, shows that HRVs prevail throughout the whole year, which is supported by a recent 10 years active surveillance study of respiratory viruses performed in Germany (105). The increased testing for respiratory viral infections during the 2009 H1N1 IfA pandemic revealed that several of the samples that were referred to the laboratory for H1N1 IfA testing were in fact positive for other viruses, mainly HRV, as shown in figure 9. This supports the use of multiplex assays in this setting.

4.8 Rhinovirus heterogeneity (paper IV)

HRV was the most commonly found respiratory virus in this study (paper IV), and is known to cause approximately half of all URTIs. HRV was also found to occur throughout the year, without the common characteristic of many other respiratory viruses that decrease significantly, or in fact disappear totally, during summer. One explanation for the endemic pattern seen in HRV infections could be associated with the plethora of different serotypes that characterizes this virus. HRV and EV, both belonging to the *Picornaviridae family*, display great biodiversity with a multitude of subtypes (>100 subtypes for HRV and 71 subtypes for EV), and the two viruses can be difficult to distinguish from each other using multiple PCR tests.

The increasing use of NAATs has revealed the existence of a novel species of HRV, which has been named HRV-C. This species may be novel to the medical community but has in fact been circulating for more than 250 years and has a worldwide distribution (197). Furthermore HRV-C has been reported to occur year around and have a similar seasonal distribution to other HRV species (193).

To further investigate the heterogeneity of HRV we sequenced 59 samples (17 samples from 2006-2007, 9 from 2008, and 33 from 2009-2010) positive for HRV and with a relatively high viral load (Ct-value <30). Phylogenetic analysis, see figure 11 (following page), revealed that 32 (54%) belonged to HRV type A (HRV-A), one (2%) to HRV-type B (HRV-B), 25 to HRV-C (42%), and one (2%) to EV-B (Coxsackie A9).

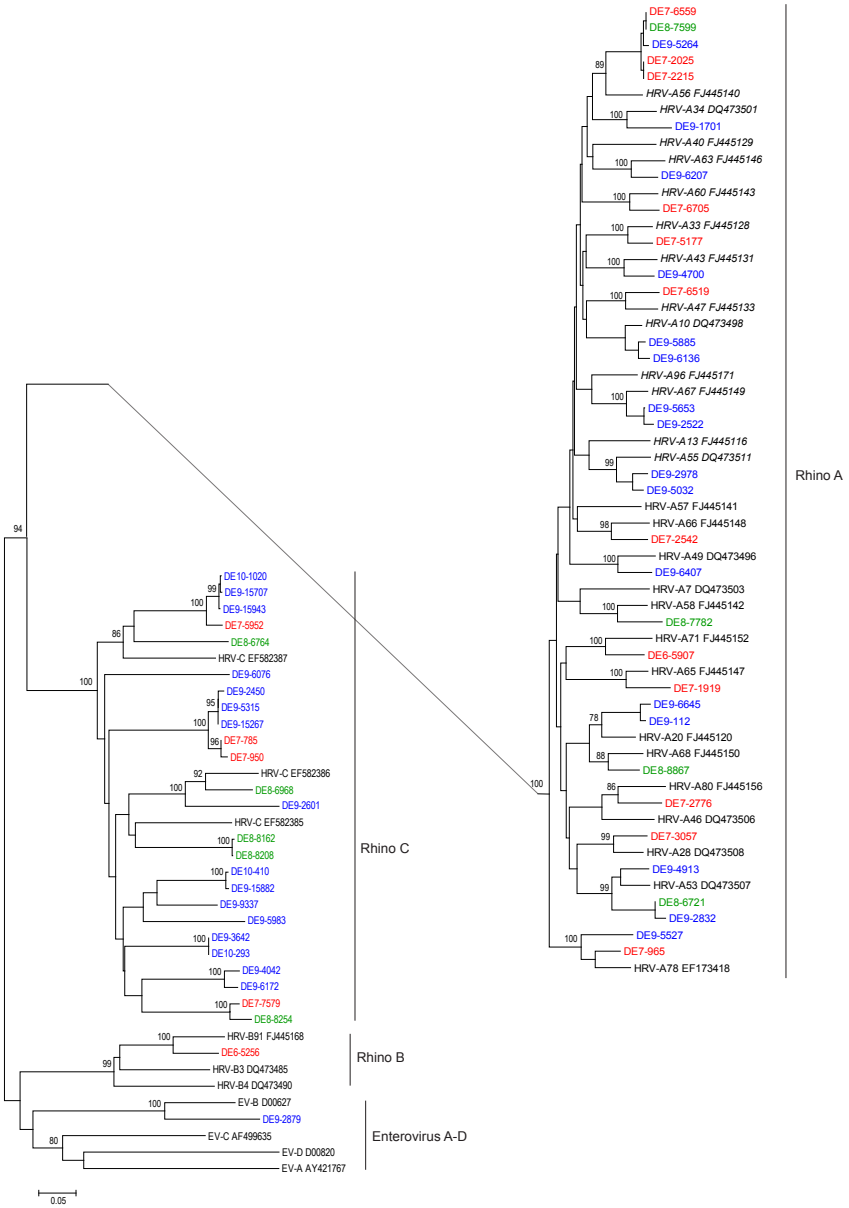

Figure 11: Neighbour-joining tree from distance matrix analysis of a 430 nt long part of the VP2-VP4 region of rhinovirus using the MEGA4 software. Bootstrapping to 500 replicates was applied and bootstrap values above 60 are shown. The analysis included 59 HRV sequences obtained in the present study and a selection of reference strains (in black), representing relevant subtypes. A Coxsackie A10 (enterovirus group A) sequence was used as outgroup. Colour coding represents different seasons samples were collected; red colour depicts 2006-2007, green colour depicts 2008 and blue colour depicts 2009-2010.

The genetic variability within HRV-C was even higher than within HRV-A; the median genetic distance was 0.25 between HRV-C strains and 0.20 between HRV-A strains ($p<0.001$). The predominance of HRV-A and HRV-C is in line with a recent report by Wisdom et al (198).

The same group (193) further described a rapid turnover of HRV variants during the observed seasons. However, in our study (covering 36 months compared to 13 months in the study by Wisdom et al.) the same HRV subtypes appeared during several seasons, as depicted in figure 11. The extensive genetic variability within each subtype, and within the time periods studied, supports simultaneous existence of multiple HRV-subtypes in the community at any given time point. This in turn may facilitate for HRV to evade herd immunity and to prevail during seasons with relatively low incidence, such as the summer months.

In summary, the robust nature of this non-enveloped virus, coupled with high genetic variability and a relatively long time period of viral shedding (as described in paper II), may explain why HRVs dominate as the cause of URTIs and prevail across seasons.

5 CONCLUSIONS

- The real time multiplex PCR method investigated proved to be reliable for the detection of various respiratory pathogens

- Detection of respiratory pathogens in nasopharyngeal swab specimens is dependent on the duration of symptoms, which is probably related to a gradual decrease of viral shedding over time

- Access to a rapid etiologic diagnostic tool for detection of respiratory viruses can reduce antibiotic overuse in primary care, but probably needs to be combined with a predefined algorithm for antibiotic stewardship to generate a sustained result

- Most respiratory tract infections display seasonal variation and detection rate using the multiplex assay depend on season and age, but the proportion of positive tests exceed 30% at all times. This finding supports the use of similar diagnostic methods throughout the year

5.1 Concluding remarks

The findings of this thesis support the use of similar diagnostic tests as the multiplex PCR evaluated in routine care for diagnosing acute respiratory tract infections. The method provides useful information on the aetiology of these infections for the treating physician, in a high frequency all year around. Both season and age of the patient affects the detection rate of the analysis, and should be taken into account when interpreting the result of the test. The use of this test also provides important information in understanding the epidemiology of respiratory infections as well as for infection control.

It is important to keep in mind that genome detection methods, like the one evaluated in this thesis, detects only the specific gene sequence of the microbial agent that the primers are designed to bind to. If the genome of the agent has mutated in such a way that it affects the gene sequence targeted, no amplification will occur and the analysis will turn out false negative. In order to avoid this the primers of all agents are directed at a conserved region of the genome. Also, a virus or a bacterium that is not included in the panel, but may be present in the patient sample, is not detected. Virus isolation or culture however, would probably detect agents that are not initially suspected. Other methods of genome detection, such as mass sequencing and the use of random primers, avoid this dilemma but these techniques are so far too costly and too cumbersome for use in clinical routine.

Using the multiplex PCR method, or similar methods, may reduce antibiotic prescription rates at initial visit, in adult patients with ARTI in an outpatient setting. This effect is probably not sustained unless combined with some kind of antibiotic stewardship including a management and follow-up plan. The use of CRP to distinguish between viral and bacterial ARTIs in this setting does not seem to guide the treating physicians to any great extent, at least not when the probability of pneumonia is low (which was the case in the majority of our study population). This finding is in line with the current recommendations of the use of CRP in respiratory infections (as is described in the guidelines from the Medical Products Agency, 2007), and etiologic diagnosis, as best achieved by multiplex PCR or similar methods, is probably most efficient in the group of patients with a CRP >50mg/L, and with an uncertain diagnosis, as a way to reduce and maintain a low prescription rate of antibiotics in uncomplicated ARTIs.

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