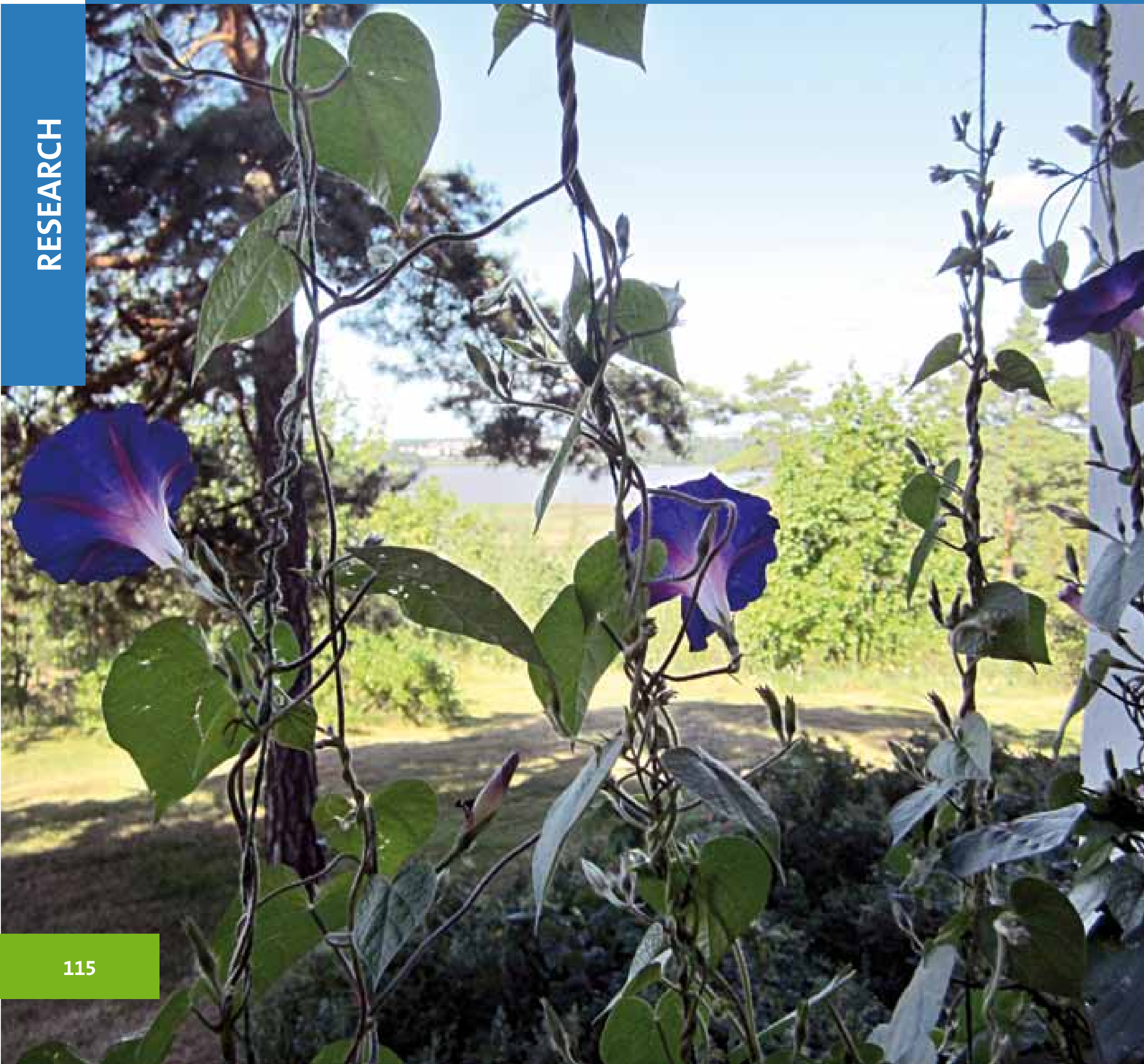
NATIONAL INSTITUTE FOR HEALTH AND WELFARE

Riitta Räty

Laboratory Diagnosis and Surveillance of Acute Respiratory Tract Infections Caused by Certain Common Viruses and *Mycoplasma pneumoniae*

RESEARCH



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RESEARCH 115

Riitta Räty

Laboratory Diagnosis and Surveillance of Acute Respiratory Tract Infections Caused by Certain Common Viruses and *Mycoplasma pneumoniae*

ACADEMIC DISSERTATION

To be presented with the permission of the Faculty of Biological and Environmental Sciences of the University of Helsinki for public examination in the auditorium 1041 of Biocenter 2, Viikinkaari 5, Helsinki on November 29th 2013, at 12 o'clock noon

National Institute for Health and Welfare
Department of Infectious Disease Surveillance and Control
Virology Unit
and

University of Helsinki
Faculty of Biological and Environmental Sciences
Department of Biosciences

Helsinki 2013

NATIONAL INSTITUTE
FOR HEALTH AND WELFARE

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Cover photo: Riitta Räty and *Ipomoea purpurea*

ISBN 978-952-302-006-1 (printed)
ISSN 1798-0054 (printed)
ISBN 978-952-302-007-8 (pdf)
ISSN 1798-0062 (pdf)

URN:ISBN:978-952-302-007-8
http://urn.fi/URN:ISBN: 978-952-302-007-8

Juvenes Print – Suomen Yliopistopaino
Helsinki, Finland 2013

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Dedicated to
Muru and Toivo

Abstract

Riitta Räty. Laboratory Diagnosis and Surveillance of Acute Respiratory Tract Infections Caused by Certain Common Viruses and *Mycoplasma pneumoniae*. National Institute for Health and Welfare (THL). Research 115. 111 pages. Helsinki, Finland 2013
ISBN 978-952-302-006-1 (printed); ISBN 978-952-302-007-8 (pdf)

Respiratory infections are the most common illnesses worldwide, and a large fraction of those are of viral origin. These infections are associated with significant morbidity and mortality as well as substantial socio-economic impact. Rapid identification of a causing agent provides guidance for treatment decisions and may help prevent the further spread of the microbe.

Adenoviruses frequently cause outbreaks of respiratory infections in army training centers. Different laboratory methods, including virus antigen detection by IF, TR-FIA and EIA, conventional culture, rapid culture, PCR, and serology by CFT, have been applied for diagnosing adenovirus infections in military conscripts. In that study the diagnostic efficacy of different methods was compared. An almost equal number of diagnoses was achieved by PCR and by virus culture, while virus antigen detection by EIA and TR-FIA was considerably less sensitive. Also sero-diagnosis by CFT identified significant rises of antibody titers in patients who remained negative by virus antigen tests.

Acute otitis media is a common complication of upper respiratory tract infection in young children. The role of *Mycoplasma pneumoniae* in the etiology of AOM is poorly understood. A PCR for the partial amplification of the P1 gene was developed and applied for the detection of *M. pneumoniae* in NPA and MEF specimens from children with AOM. Antibodies to *M. pneumoniae* were measured by CFT and by EIA. In 4 % of the MEF specimens *M. pneumoniae* was detected by PCR. Serological results did not consistently support the PCR findings.

The Finnish Otitis Media (FinOM) study provided an excellent opportunity to determine the etiology of this disease and to evaluate different laboratory diagnostic methods. From children presenting with AOM, NPA, MEF, as well as acute and convalescent phase serum were collected. NPAs and MEFs were tested for the presence of respiratory virus antigens by TR-FIA. Antibodies to the same viruses were measured by CFT or EIA. In one third of the cases a viral etiology could be established by antigen detection and/or by serology. RSV was the most frequently identified virus in this material. In this study, significantly more diagnoses were established by serology than by direct virus antigen detection.

The quality of the clinical specimen greatly influences the sensitivity of laboratory diagnosis. In an attempt to evaluate the optimal specimen for the detection of *M. pneumoniae* by PCR in patients with X-ray confirmed pneumonia, sputum, throat swab, and NPA were collected from hospitalized patients. In addition, acute and convalescent phase serum specimens were obtained. In this comparison, sputum has

been found to be the best respiratory specimen. In two thirds of the patients from whom all specimens were available, *M. pneumoniae* could be detected by PCR in the sputum. Serological findings well supported the results obtained by PCR.

In a long-term surveillance of respiratory infections in certain segments of the Finnish population, 7 common respiratory viruses were monitored by antigen detection using TR-FIA. From many patients, also acute and convalescent phase serum specimens were available for measurement of antibodies by CFT. Over a period of 16 years, influenza epidemics have been observed each winter. While RSV was the predominant virus in small children, adenoviruses were the most frequently identified pathogen in army recruits. Although serological results are rarely available during the acute phase of a respiratory infection, also in this study, more diagnostic findings were obtained by CFT as compared to virus antigen detection.

Keywords: respiratory infections, respiratory viruses, *M. pneumoniae*, clinical specimen, laboratory diagnosis, antigen detection, PCR, virus culture, serology

Tiivistelmä

Riitta Räty. Akuuttien hengitystieinfektioiden laboratoriodiagnostiikka muutaman tärkeimmän viruksen ja Mycoplasma pneumoniae-bakteerin valossa sekä näiden infektioiden epidemiologisesta seurannasta. Terveyden ja hyvinvoinnin laitos (THL). Tutkimus 115. 111 sivua. Helsinki, Suomi 2013.
ISBN 978-952-302-006-1 (painettu); ISBN 978-952-302-007-8 (pdf)

Hengitystieinfektiot ovat maailman yleisimpiä sairauksia ja suurin osa niistä on virusten aiheuttamia. Näihin infektioihin liittyy suuri sairastavuus ja merkittävä kokonaiskuolleisuus, joten niiden yhteiskunnallinen ja taloudellinen vaikutus voi olla huomattava. Aiheuttajan nopea tunnistaminen auttaa valitsemaan oikeat hoitotoimenpiteet ja vähentämään infektion leviämistä ympäristöön.

Adenovirukset aiheuttavat usein hengitystie-infektioryppäitä varuskunnissa. Varusmiesten adenovirusinfektioiden diagnostisoimiseksi on kehitetty useita eri laboratoriomenetelmiä kuten virusantigeenidetektio IF:lla, TR-FIA:lla tai EIA:lla, perinteinen virusviljely, pikaviljely, PCR ja virusserologia CF-testillä. Tässä työssä näiden menetelmien diagnostista ottavuutta verrattiin toisiinsa. Suurin piirtein yhtä paljon diagnooseja tehtiin PCR:llä ja virusviljelyllä kun taas antigeenidetektio TR-FIA:lla ja EIA:lla olivat paljon epäherkempiä. Myös CF-serologia löysi merkittäviä vasta-ainenousuja potilailla, jotka jäivät antigeenidetektiossa negatiivisiksi.

Akuutti välikorvantulehdus on yleinen hengitystieinfektion komplikaatio lapsilla. *M.pneumoniaen* osuudesta välikorvantulehduksissa on ollut hyvin vähän tietoa. Tässä työssä kehitettiin PCR-menetelmä, jolla voitiin monistaa osa P1-geeniä ja sovellusta käytettiin *M.pneumoniaen* havaitsemiseksi korvatulehduslasten NPA ja MEF-näytteistä. *M.pneumoniae*-vasta-aineita etsittiin CF- testillä ja EIA:lla. Neljä prosenttia MEF-näytteistä oli positiivisia *M.pneumoniaen* suhteen PCR:llä. Serologian ja PCR-testin tulokset olivat vain pieneltä osalta yhteneväisiä.

FinOM-tutkimus tarjosi erinomaisen tilaisuuden tutkia välikorvatulehdusten mikrobietiologiaa ja eri menetelmien diagnostista ottavuutta. Lapsilta, joilla välikorvantulehdus todettiin, otettiin NPA- ja MEF-näytteet sekä kerättiin pariverinäytteet (akuutin ja toipilasvaiheen verinäytteet). NPA ja MEF-näytteistä tutkittiin virusantigeenit TR-FIA:lla. Vasta-aineet näitä viruksia vastaan tutkittiin CF-testillä. Kolmasosassa tapauksista voitiin virusetiologia todeta joko antigeenidetektiolla tai serologisesti. RSV oli yleisin viruslöydös tässä aineistossa. Diagnooseja tehtiin huomattavasti enemmän serologisen testin avulla kuin antigeenidetektiolla.

Näytetyyppi vaikuttaa merkittävästi diagnostisten testien herkkyyteen. Potilailta, joilla oli röntgenologisesti todettu keuhkokuume, kerättiin yskös, imulima ja nenänielutikkunäyte sen arvioimiseksi mikä näistä näytetyypeistä olisi ihanteellisin *M.pneumoniaen* PCR-diagnostiikkaa varten. Lisäksi potilailta kerättiin myös pariseeruminäytteet. Vertailu osoitti, että *M.pneumoniae* löytyi useimmin yskösnäytteestä PCR:llä. Serologian ja PCR-testin tulosten yhteneväisyys oli hyvä.

Pitkän aikavälin hengitystieinfektioiden seurantaan osallistui eri väestönosia Suomessa ja seurannan aikana haettiin antigeenidetektion (TR-FIA) avulla seitsemän tavallista hengitystievirusta. Suuresta osasta tutkittavia oli myös otettu pariseeruminäytteet ja niistä tutkittiin mahdolliset mikrobivasta-ainetasojen nousut CF-testin avulla. Kuudentoista vuoden aikana on joka talvi esiintynyt influenssaepidemioita. RSV oli vallitseva virus pikkulapsilla, kun taas adenovirus oli yleisin varusmiehillä tavattu patogeeni. Vaikka serologisia tuloksia harvoin on käytössä hengitystieinfektion akuutissa vaiheessa, löytyy serologian avulla usein, kuten tässäkin tutkimuksessa, enemmän diagnooseja CF-testillä kuin virusantigeenidetektiolla.

Avainsanat: hengitystieinfektiot, respiratoriset virukset, *M. pneumoniae*, kliiniset näytteet, laboratoriodiagnostiikka, antigeenidetektio, PCR, virusviljely, serologia

Sammandrag

Riitta Räty. Diagnostisering och övervakning av akuta respiratoriska infektioner orsakade av de sju vanligaste respiratoriska virusen och *Mycoplasma pneumoniae*. Institutet för hälsa och välfärd (THL). Forskning 115. 111 sidor. Helsingfors, Finland 2013.
ISBN 978-952-302-006-1 (tryckt); ISBN 978-952-302-007-8 (pdf)

Luftvägsinfektioner är de vanligaste smittsamma sjukdomarna i hela världen, och en stor del av dem är av viralt ursprung. Dessa infektioner är förknippade med hög sjuklighet och dödlighet, och kan ha betydande socioekonomiska effekter. Snabb identifiering av den orsakande patogenen ger vägledning för behandlingsbeslut och kan bidra till att förhindra ytterligare spridning av patogenen.

Adenovirus orsakar ofta utbrott av luftvägsinfektioner i arméns utbildningscenter. Olika laboratoriemetoder, inclusive virusantigen påvisning med IF, TR-FIA eller EIA, konventionell kultur, snabbkultur, PCR och serologi med CFT, har tillämpats för att diagnostisera adenovirus-infektioner hos de värnpliktiga inom armén. Den diagnostiska effektiviteten hos dessa metoder jämfördes med varandra. Ett nästan lika stort antal diagnoser uppnåddes med PCR och med virusodling, medan virusantigen påvisning med EIA eller TR-FIA var avsevärt mindre känslig. Sero-diagnos genom CFT identifierade betydande ökningar av antikroppstitrar hos patienter vars prov i virusantigen-tester gav negativa resultat.

Akut öroninflammation (AOM) är en vanlig komplikation vid övre luftvägsinfektion hos små barn. Vilken roll *M. pneumoniae* spelar i etiologin av AOM förstår man ännu ganska dåligt. En PCR utvecklades och tillämpades för detektion av *M. pneumoniae* för amplification av en del av P1-genen i NPA- och MEF-prov från barn med AOM. Antikroppar mot *M. pneumoniae* mättes genom CFT och PCR. I 4 % av MEF-proven detekterades *M. pneumoniae* med PCR. Serologiska resultat stödde inte PCR resultaten.

Den finska öroninflammationsstudien (FinOM) gav ett utmärkt tillfälle för att bestämma etiologin av denna sjukdom och för att utvärdera olika laboratoriediagnostiska metoder. Från barn som uppvisade AOM, samlades NPA, MEF samt blodprov under den akuta fasen och konvalescensfasen. NPA- och MEF-proven testades med avseende på förekomst av respiratoriska virusantigener med TR-FIA. Antikroppar mot samma virus mättes genom CFT eller EIA. I en tredjedel av fallen kunde en viral etiologi konstateras genom antigendetektion och/eller serologi. RSV var det oftast identifierade viruset i materialet. I denna studie fanns betydligt fler diagnoser som konstaterats genom serologi än genom direkt upptäckt av virusantigen.

Kvaliteten på kliniska prov påverkar laboratoriediagnosens känslighet i hög grad. I ett försök att utvärdera den optimala provtypen för detektion av *M. pneumoniae* med PCR hos patienter med en röntgenbekräftad lunginflammation, samlades sputum, näs-hals och NPA-prov. Dessutom samlades blodprov under den

akuta fasen och konvalescens-fasen. I denna jämförelse var sputum det bästa respiratoriska provet. I två tredjedelar av patienter från vilka alla prov var tillgängliga, detekterades *M. pneumoniae* från sputum. Serologiska resultaten stödde väl PCR resultaten.

I en långsiktig bevakning av luftvägsinfektioner hos vissa befolkningsgrupper i Finland, söktes sju vanliga respiratoriska virus med antigendetektion (TR-FIA). Från en stor andel patienter fanns det också serumprov som tagits under den akuta fasen och konvalescentfasen, som kunde användas för mätning av antikroppar med CFT. Under en period på sexton år, har influensaepidemier observerats varje vinter. Medan RSV var det dominerande viruset hos små barn, var adenovirus den oftast identifierade patogenen hos de värnpliktiga. Även om serologiska resultat sällan är tillgängliga under den akuta fasen av luftvägsinfektioner, ger de ofta, likt i denna studie, mera diagnostiska resultat genom CFT jämfört med påvisning av virusantigen.

Nyckelord: luftvägsinfektioner, kliniska prov, repiratoriska virus, *M. pneumoniae* laboratoriediagnostik, antigen, PCR, virus kultur, serologi

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Abbreviations

AGE	Agarose gel electrophoresis
AOM	Acute Otitis Media
ARI	Acute Respiratory Infection
CDC	Center for Disease Control
CFT	Complement fixation test
ECDC	European Center for Disease Control
EIA	Enzyme immunoassay
FAO	UN Food and Agriculture Organization
GISRS	Global Influenza Surveillance and Response System
IF, IFA	Immunofluorescence based assays
ILI	Influenza Like Illness
MEF	Middle ear fluid
NIC	National Influenza Center
NPA	Nasopharyngeal aspirate
OIE	World Organization for Animal Health
PCR	Polymerase chain reaction
RSV	Respiratory syncytial virus
TR-FIA	Time resolved fluoroimmunoassay
WHO	World Health Organization

1 Introduction

Respiratory infections are the most common illnesses worldwide, causing significant morbidity and mortality. The clinical picture can vary from mild, even subclinical to severe, sometimes fatal infection. Acute upper respiratory infections are often of viral etiology. Many of the causing agents are readily transmitted from human to human and thus often cause sizeable epidemics that may be limited locally or can spread nationally or internationally. Laboratory diagnosis and surveillance of respiratory infections are crucial for many reasons. Rapid identification of the causing agent can direct decisions concerning optimal antimicrobial or symptomatic treatment strategies. The recognition of the causative agent may help prevent further spread of the pathogen by guiding decisions about cohorting individuals infected with the same pathogen or by reducing social contacts. Through detailed characterization of influenza viruses, suitable candidate vaccine viruses can be identified. Laboratory-based diagnosis and surveillance applying conventional and molecular methods can help identify new, previously unknown or unidentified pathogens. It also can assist early recognition of significant changes in a certain agent, such as the sudden emergence of drug-resistant influenza viruses, influenza viruses with uncommon characteristics, or changes that may alter the virulence or pathogenicity of a virus. Laboratory diagnostic results help understand the epidemiology and clinical characteristics of certain pathogens.

Although many clinical laboratories offer testing for several respiratory pathogens, national and international surveillance is usually limited to influenza A and B viruses, and to some other viruses. Over a period of 16 years, from 1989 to 2005, it was attempted to establish a microbiological etiology for 12,020 patients with respiratory infection. Samples were analyzed for adenoviruses, influenza virus types A and B, parainfluenza virus types 1 – 3, respiratory syncytial virus (RSV), and *M. pneumoniae*. Viral antigens were detected using time-resolved fluoroimmunoassays (TR-FIA) and antibodies to all pathogens mentioned were measured with complement fixation test.

2 Review of the Literature

2.1 Novel respiratory Viruses

Using modern diagnostic laboratory methods, an etiological agent can be detected in almost every patient presenting with signs and symptoms of an acute respiratory tract infection. Many of the common respiratory viruses have been identified between the 1930s and 1950s, and their clinical and epidemiological significance has been well established. Occasionally new respiratory pathogens are introduced into the human population by transmission of animal viruses to humans. In addition, with novel, molecular methods that have become available during recent years, new or previously unrecognized viruses have been identified (Table 1).

Middle East Respiratory Syndrome Coronavirus (MERS-Coronavirus). As recently as in summer 2012, a 60-year old man in Saudi-Arabia was hospitalized due to an acute severe pneumonia and subsequent kidney failure. From a sputum sample taken early after hospital admission, a novel coronavirus, likely originating from bats, was identified (Anderson and Baric 2012; Zaki, van Boheemen et al. 2012). A second patient transferred from the Middle East to the United Kingdom in the fall of 2012, harboured almost an identical virus in his respiratory secretions (Bermingham, Chand et al. 2012). Within a very short time, specific diagnostic tests were developed and control reagents were made available to diagnostic laboratories (Corman, Eckerle et al. 2012).

Coronaviruses NL63 and HKU1. The SARS epidemic stimulated intense research of coronaviruses and within a short period of time two new coronaviruses, coronavirus NL-63 and coronavirus HKU1, were detected (van der Hoek, Pyrc et al. 2004; Woo, Lau et al. 2005). Both of these viruses have evidently been human pathogens for many years and it is now understood that these viruses cause acute respiratory infections (Jartti, Jartti et al. 2012).

SARS coronavirus. The occurrence of MERS-coronavirus generated fears that a similar epidemic as that caused by SARS coronavirus in 2003 might evolve. In southern China cases of atypical pneumonia were observed in individuals who had contact with civet cats late in 2002. After the epidemic spread to Hong Kong, the causative agent of the illness was rapidly identified as a previously unknown coronavirus (Drosten, Gunther et al. 2003; Ksiazek, Erdman et al. 2003; Peiris, Lai et al. 2003; Tsang, Ho et al. 2003). Over the following weeks and months, the epidemic spread to 26 countries, and over 8000 cases including 774 deaths have been observed (Peiris, Yuen et al. 2003). Rapid, effective public health measures and close international scientific cooperation enabled the successful containment of the epidemic already in summer of 2003 and the outbreak ended (Peiris, Yuen et al. 2003). It is believed that also the SARS-coronavirus originated from bats (Wang, Shi et al. 2006).

Influenza A(H7N9). Just recently, in March 2013, severe respiratory infections with multiple deaths occurred in Eastern China around the Shanghai area. The causative agent was rapidly identified and it was found to be triple assortant virus. The virus likely obtained the H7 gene from a duck H7N3 virus, the N9 gene from a Korean H7N9 virus, and the rest of the genes from an avian H9N2 virus (Gao, Lu et al. 2013; Gao, Cao et al. 2013). By April 18, 2013, altogether 83 human cases have been identified with 17 deaths (http://www.chinacdc.cn/en/research_5311).

Influenza A (H3N2v; H1N2v; H1N1v). Between August 2011 and October 2012, 319 human infections with a swine-origin H3N2 influenza virus (H3N2v) have been reported to the Centers for Disease Control and Prevention, Atlanta http://www.cdc.gov/flu/swineflu/h3n2v-case-count.htm (accessed November 7, 2012). This virus was first transmitted from pigs to humans in the summer of 2011 in the United States. Most infected individuals have been in close contact with pigs, particularly at agricultural fairs. After several months of absence, the virus reoccurred in summer 2012 in several states of the USA. Meanwhile the virus had obtained through reassortment the gene segment 7 coding for the M1 and M2 proteins from the influenza A(H1N1)pdm09 virus, i.e., the swine origin virus that caused the pandemic in 2009 http://www.cdc.gov/flu/spotlights/h3n2v_us_cases.htm (accessed November 7, 2012). Most of these human cases have been relatively mild and there is little evidence for human-to-human transmission. In addition to these cases caused by the influenza A(H3N2v) strain, also a few human cases of H1N2v and H1N1v have been reported. These influenza viruses of swine origin could present a pandemic threat, particularly if they should gain the ability to easily spread among humans. In some studies it has been shown, however, that certain segments of the population, particularly individuals >10 years of age, have cross-reactive antibodies against the H3N2v virus which may confer some protection (http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6114a1.htm) (accessed November 7, 2012) (Waalen, Kilander et al. 2012). This H3N2v virus has obtained its hemagglutinin gene from a human virus that has been transmitted to pigs in the mid 1990s (Webby, Swenson et al. 2000).

Influenza A triple-reassortant. Similar incidences of influenza virus transmission from pig to human have occurred previously. Triple-reassortant influenza A virus of the subtype H1N1 became enzootic in North American pigs in the late 1990s (Shinde, Bridges et al. 2009). After multiple reassortment events, this virus had 5 gene segments (HA, NP, NA, M, NS) from the classic North American swine lineage, 2 from the avian North American lineage (PB2, PA), and the PB1 gene segment from a seasonal human influenza A(H3N2) virus. Between December 2005 and February 2009, this virus has caused 10 sporadic cases in humans in the United States. Most of these patients showed symptoms of typical influenza infection, two of them had X-ray confirmed pneumonia and all patients recovered (Shinde, Bridges et al. 2009).

Influenza A(H1N1)pdm09. Before evolving into a pandemic virus, this triple reassortant virus exchanged two gene segments (NA and M) with those of a virus belonging to the Eurasian swine lineage (Dawood, Jain et al. 2009; Garten, Davis et al. 2009; Trifonov, Khiabanian et al. 2009; Zimmer and Burke 2009). This virus was first identified in two Californian children in April 2009 (MMWR 2009) and rapidly spread to many countries. On June 11, 2009, the Director-General of WHO, Dr. Margaret Chan, declared the beginning of the 2009 influenza pandemic (http://www.who.int/mediacentre/news/statements/2009/h1n1_pandemic_phase6_20090611/en/index.html) (accessed November 8, 2012). August 10, 2010, again, Dr. Chan informed that the world is no longer in phase 6 of influenza pandemic alert. In other words, the pandemic was over http://www.who.int/mediacentre/news/statements/2010/h1n1_vpc_20100810/en/index.html). During the pandemic, approximately 18,500 laboratory-confirmed deaths caused by the pandemic virus were reported to WHO. However, this seems to represent only the tip of the iceberg, as later estimates based on modeling studies indicate that the mortality rate may have been at least 15-times higher (Dawood, Iuliano et al. 2012).

Influenza A(H5N1). In May 1997, a 3-year-old child in Hong Kong died of multiple complications during an acute respiratory illness. An influenza A virus was isolated from a tracheal aspirate collected from the patient 10 days after the onset of symptoms (MMWR 1997(a)). The virus was sent to reference laboratories for further characterization and it was identified to be an influenza A virus of subtype H5N1 (Claas, Osterhaus et al. 1998; Subbarao, Klimov et al. 1998; Yuen, Chan et al. 1998). Before this incidence, the subtype H5N1 of influenza A virus has never been isolated from humans. At the same time, a major outbreak of avian influenza caused by this highly pathogenic H5N1 virus was observed in the Hong Kong area. Several more individuals contracted infection with this virus later during the same year (MMWR 1997(b)). Swift stamping out measures brought the outbreak in birds to a halt and no further human cases were observed for several years. However, in early 2003 the same virus re-emerged in the Hong Kong area (Peiris, Yu et al. 2004) and subsequently spread to other countries (Hien, de Jong et al. 2004). This set off a world-wide public health response as, under the leadership of WHO and other organizations, countries started to prepare for a pandemic possibly caused by this virus. Until March 31, 2013, this virus caused 622 laboratory-confirmed human cases in Azerbaijan, Bangladesh, Cambodia, China, Djibouti, Egypt, Indonesia, Iraq, Lao People's Democratic Republic, Myanmar, Nigeria, Pakistan, Thailand, Turkey, and Viet Nam. Overall, 371 (60 %) of these cases have been fatal. (www.who.int/influenza/human_animal_interface/EN_GIP_20130312CumulativeNumberH5N1cases.pdf). Luckily this virus has not yet acquired the ability to easily spread from human to human. However, in animal experiments, it has been recently shown that a few mutations in several genes can render the virus more transmissible

between ferrets, an animal frequently used for studies on human influenza (Herfst, Schrauwen et al. 2012; Imai, Watanabe et al. 2012).

Influenza A(H7N7). In 2003, another highly pathogenic avian influenza virus, influenza A(H7N7), caused a massive outbreak in chicken breeding farms of The Netherlands. It has been estimated that more than 4,000 individuals have been involved in culling infected birds, cleaning and disinfecting breeding facilities and other activities. The H7N7 virus has been detected from 86 of those persons. One of them, a veterinarian examining ill animals, died from this infection, all others experienced relatively mild respiratory symptoms or conjunctivitis. In a few cases, the virus was transmitted from poultry workers to their family members (Fouchier, Schneeberger et al. 2004; Koopmans, Wilbrink et al. 2004).

Influenza A (H9N2, H10N7). In rare instances also other subtypes of influenza A have been transmitted to humans. Influenza A(H9N2) is widespread in Asian poultry. Two human cases have been described in Hong Kong in 1999 (Peiris, Yuen et al. 1999). A few human cases with an influenza A(H10N7) virus have been reported from Egypt (http://new.paho.org/hq/dmdocuments/2010/Avian_Influenza_Egypt_070503.pdf) and from Australia (Arzey, Kirkland et al. 2012).

Influenza A(H17). As an interesting curiosity, evidence for an infection with an influenza virus has recently been found in bats sampled in Guatemala. The hemagglutinin of these viruses is distinct from the 16 subtypes identified so far and it has been designated as H17 (Tong, Li et al. 2012). It is speculated that this virus might be a precursor of the current influenza A viruses.

Hantavirus pulmonary syndrome, Sin nombre virus. During the first months of 1993, several patients with an influenza-like illness which rapidly progressed to respiratory failure were seen in the Four Corners area of New Mexico, Arizona, Colorado and Utah (MMWR 1993; MMWR 2010). Based on the clinical picture, 24 cases, including 12 deaths, were reported by the first week of June, and some of the cases have been closely related, even living in the same household, which indicated an infectious cause. All laboratory tests for known respiratory pathogens yielded negative results (Duchin, Koster et al. 1994). Serologic testing of a wide range of pathogens suggested a possible involvement of a hantavirus. Based on this finding it was possible to amplify hantavirus specific gene fragments by PCR from tissue specimens obtained from patients and from deer mice (Peromyscus maniculatus) that were trapped in the area, and the entire nucleotide sequence of the virus could be established. It turned out that this illness was caused by a new hantavirus, and the sequences obtained from patients and from animals were identical (Nichol, Spiropoulou et al. 1993). The illness was named hantavirus pulmonary syndrome (HPS). In summer 2012, 6 cases of HPS were identified among the visitors of the Yosemite National Park in California. Two of these individuals died (Rhee, Clark et al. 2012; WER 2012).

Rhinovirus C. During the influenza season 2004 – 2005, laboratories in New York received a number of clinical samples from patients with influenza-like illness, which remained negative when tested for influenza and other respiratory viruses. Applying MassTag PCR technology, a significant fraction of these previously negative specimens was found to contain rhinoviruses. From these rhinovirus-positive samples the nucleotide sequence of the PCR-amplified product was determined. Phylogenetic analysis revealed that some of these rhinoviruses formed a unique clade between the rhinovirus clades A and B (Lamson, Renwick et al. 2006). These results thus revealed a new group of rhinoviruses, subsequently termed rhinovirus C (Lau, Yip et al. 2007). Using specific PCR methods for this rhinovirus, it was soon recognized that this virus was found in many parts of the world (Briese, Renwick et al. 2008) and it is able to cause severe respiratory infections particularly in children (Renwick, Schweiger et al. 2007; Khetsuriani, Lu et al. 2008; Peltola, Jartti et al. 2009). Rhinoviruses are commonly found in respiratory secretions and in middle ear fluids of children with acute otitis media, and also rhinovirus C was found in such specimens (Savolainen-Kopra, Blomqvist et al. 2009).

Metapneumovirus. Over a period of 20 years, virologists in The Netherlands have identified morphological changes in tertiary cultures of monkey kidney cells inoculated with human respiratory specimens. With conventional methods and available reagents, no cause for this finding could be determined. However, electron microscopic examination of supernatants of the cultures revealed the presence of paramyxovirus-like particles. When a random PCR amplification was performed, 20 different bands were identified for the unknown virus, and the nucleotide sequence of these amplification products was determined. The sequences found most closely resembled those of an avian pneumovirus. With newly designed, specific primers, it was possible to obtain the entire genome of this novel virus, which was then named human metapneumovirus (van den Hoogen, de Jong et al. 2001). The authors found that by age five, virtually all tested individuals had antibodies against this novel virus, and concluded that this virus has been a human pathogen for a long period of time. Studying retrospectively respiratory specimens collected from patients of all age with respiratory tract infections in Canada, hMPV was identified as a pathogen (Boivin, Abed et al. 2002). The authors reported that pneumonitis and bronchiolitis were frequent diagnoses in hMPV-infected, hospitalized children, while the most common diagnoses in elderly subjects were bronchitis and/or bronchospasm and pneumonitis. In a prospective study of young and older adults hospitalized for cardiopulmonary illnesses, 44 of 984 individuals had evidence of infection with hMPV (Falsey, Erdman et al. 2003).

Polyomaviruses KI and WU. In 2007, two groups, one in Sweden (Allander, Andreasson et al. 2007) and one in Australia (Gaynor, Nissen et al. 2007) detected two previously unknown polyomaviruses in respiratory specimens using sophisticated molecular methods (polyomavirus KI; polyomavirus WU). The clinical relevance of these two viruses remains unclear. One group in Germany has found

these viruses in nasopharyngeal aspirates and bronchoalveolar lavage specimens in 3 of 229 children hospitalized due to an acute respiratory tract infection (Mueller, Simon et al. 2009). No other respiratory viruses were detected in these children except in one immunocompromized child whose specimen was positive for cytomegalovirus, Epstein-Barr virus, and herpesvirus 6. These polyomaviruses have also been detected simultaneously with RSV and adenovirus in lower respiratory tract infection and in older, severely immunosuppressed individuals and some authors believe that there is no association between these viruses and respiratory disease (Norja, Ubillos et al. 2007).

Adenovirus 14p1. In March and April 2006 adenovirus serotype 14 belonging to the subspecies B2 was first detected at five military training centers in the United States (Metzgar, Osuna et al. 2007; MMWR 2010). Retrospective studies indicated that this virus has appeared in Oregon in 2005 (Lewis, Schmidt et al. 2009) and soon became the predominant circulating adenovirus. Many of the adenovirus 14-infected individuals required hospitalization, supplemental oxygen, intensive care treatment, and 7 of 38 patients died (Lewis, Schmidt et al. 2009). Outbreaks in US army training centers have been described (Tate, Bunning et al. 2009). Detailed genetic analysis suggests that this so called adenovirus 14p1 possibly arose through recombination of the adenovirus type 11 and 14 ancestral strains (Kajon, Lu et al. 2010).

Bocavirus. In 2005 Allander and colleagues detected a new parvovirus in nasopharyngeal aspirates from patients with respiratory tract infection (Allander, Tammi et al. 2005). They extracted nucleic acids from concentrated cell-free supernatants of these specimens, and after random PCR amplification cloned and sequenced amplified products. Phylogenetic analysis of the obtained sequences revealed the presence of a novel parvovirus, which was named bocavirus. Based on sequence information, the authors developed a PCR method specific for bocavirus and found 7 of 378 respiratory specimens to be bocavirus positive, all of those were from children and infants (Allander, Tammi et al. 2005). It soon became clear that bocavirus is a respiratory pathogen especially of young children in many parts of the world (Sloots, Whiley et al. 2008). However, bocavirus can also be found in respiratory samples collected from asymptomatic children even for prolonged periods, which also explains the fact that bocavirus is often detected together with other common respiratory viruses (Soderlund-Venermo, Lahtinen et al. 2009; Jartti, Jartti et al. 2012). To shed more light on the importance of bocaviruses during early life, Meriluoto and colleagues studied serial serum samples

Table 1. Newly identified respiratory viruses in humans during the recent 20 years from 1993 – 2013.

Virus	Original publication
MERS-Coronavirus	Zaki, van Boheemen, et al. (2012)
HKU1	Woo, Lau, et al. (2005)
NL63	van der Hoek, Pyrc, et al. (2004)
SARS coronavirus	Peiris, Lai, et al. (2003) Ksiazek, Erdman, et al. (2003)
Influenza A(H7N9)	Gao, Cao et al. (2013)
Influenza A, triple reassortant	Shinde, Bridges, et al. (2009)
Influenza A(H1N1)pdm09	MMWR (2009) Dawood, Jain et al. (2009)
Influenza A(H5N1)	Subbarao, Klimov, et al. (1998)
Influenza A(H7N7)	Koopmans, Wilbrink, et al. (2004)
Influenza A(H9N2)	Peiris,Yuen, et al. (1999)
Sin nombre	Rhee, Clark, et al. (2012).
Rhinovirus	Lamson, Renwick, et al. (2006)
Metapneumovirus	van den Hoogen, de Jong, et al. (2001)
WU and KI	Allander, Andreasson, et al. (2007) Gaynor, Nissen et al. (2007)
Adenovirus 14	Kajon, Lu, et al. (2010)
Bocavirus	Allander, Tammi, et al. (2005)

from 109 children in a cohort that was followed from infancy to an average of 8 years (Meriluoto, Hedman et al. 2012). Sampling intervals was between 3 and 6 months. On an average, seroconversion to bocavirus was detected at the age of 2.3 years. Secondary immune reactions were seen in one third of these children. Based on antibody development, it was determined that primary infection was significantly associated with respiratory illness and associated complications. Bocavirus was also

found in children hospitalized with lower respiratory tract infection and was a frequent cause of wheezing (Deng, Gu et al. 2012). In that study samples were obtained from the lower airways, and it was demonstrated that a high viral load was associated with more severe infection, longer duration of hospitalization and wheezing.

It seems that of the bocaviruses only HBoV1 is causing respiratory infections, the other bocaviruses appear to cause enteric infections. Because HBoV DNA is often found in respiratory specimens together with other respiratory viruses, a PCR positive finding alone may not be diagnostic. However, if HBoV1 DNA is found in the blood as indication of a viremic phase or if the patient has HBoV1-specific IgM antibodies and/or shows a seroconversion to this virus, the diagnosis of HBoV1 infection can be established.

2.2 “Classical” respiratory viruses

As long as medical observations have been recorded, respiratory infections have played an important role. Hippocrates has described epidemics of respiratory illnesses, which may well have been attributed to influenza. However, the concept of viruses has been established approximately 120 years ago with the first transmission experiments of the tobacco mosaic virus. Subsequently it has become possible to study viruses in experimental animals and later also in embryonated hens’ eggs. In fact, the first influenza viruses were isolated from pigs and from humans in ferrets in the 1930s. A major breakthrough in virology occurred with the development of tissue- and cell culture techniques. Using egg and cell culture isolation, the number of recognized and at least partly characterized viruses, including respiratory viruses, rapidly increased during the latter half of last century. This led to the detection of rhinoviruses, adenoviruses, parainfluenza viruses, RSV, and the “classical” coronaviruses OC43 and 229E. Modern virus classification is largely based on genetic characteristics (Table 2). Besides these typical respiratory viruses, also other viruses can cause respiratory illnesses. One example are the herpesviruses, in particular the cytomegalovirus, which can cause severe pneumonia in immunocompromized individuals. Although beyond the scope of this thesis, it must be mentioned, that bacteria, including *Mycoplasma pneumoniae* and chlamydia, fungi, and parasites are significant causes of respiratory infections, often also with lethal outcome. Also, very importantly, tuberculosis continues to be a major global health threat.

Table 2. Viruses often associated with respiratory tract infections

Virusfamily	Virus	Nucleic acid	Special characteristics
Orthomyxoviruses	Influenza A, B, C	RNA	negative sense, segmented
Hantaviruses	Sin nombre virus	RNA	negative sense, segmented
Paramyxoviruses	Parainfluenza 1 - 4	RNA	negative sense
	RSV	RNA	negative sense
	Human metapneumo-virus	RNA	negative sense
	Measlesvirus	RNA	negative sense
Picornaviruses	Rhinovirus A - C	RNA	positive sense
	Parechovirus	RNA	positive sense
	Coxsackievirus A	RNA	positive sense
	Coxsackievirus B	RNA	positive sense
	Echovirus	RNA	positive sense
Coronaviruses	Coronavirus OC43	RNA	positive sense
	Coronavirus E229E	RNA	positive sense
	SARS-coronvirus	RNA	positive sense
	Coronavirus NL63	RNA	positive sense
	Coronavirus HKU1	RNA	positive sense
	MERS-coronavirus	RNA	positive sense
Adenoviruses	Human adenovirus A - G	DNA	
Parvoviruses	Human bocavirus	DNA	
Polyomaviruses	KI polyomavirus	DNA	
	WU polyomavirus	DNA	
Herpesviruses	Cytomegalovirus	DNA	
	Herpes simplex virus 1 and 2	DNA	
	Varicella zoster virus	DNA	
	Epstein-Barr virus	DNA	
	Human herpes virus 6 - 8	DNA	

For more detailed information about virus taxonomy see: www.ictvonline.org/virusTaxonomy.asp

2.3 Surveillance of respiratory viruses

"The purpose of surveillance is to reduce the impact of disease" (John Watson, personal communication, first meeting of the European Surveillance Network, ECDC, Stockholm, 2009). Acute respiratory infections present the most common medical condition people of all ages experience worldwide. In sporadically performed health surveys, CDC gathers health information through telephone interviews. In that survey, acute conditions are defined as health problems, which restrict normal activity and/or require medical attention, with onset no longer than 3 months before the interview and the condition is not otherwise classified as chronic. The collected data is presented as acute conditions per 100 persons per year (Table 3.).

Table 3. Acute conditions per 100 persons per year, USA, 1995. All acute conditions and respiratory conditions. (Adapted from http://www.cdc.gov/nchs/data/series/sr 10/101991.pdf; accessed November 2012).

Type of condition	All ages	< 5 years	5 – 17 years	18 – 24 years	25 – 44 years	45 – 64 years	≥ 65 years
All acute conditions	174.4	364.4	236.4	158.4	156.9	119.0	103.0
All respiratory conditions	85.2	159.5	122.8	79.7	80.5	57.6	39.0
Common cold	23.1	53.7	33.0	21.8	18.6	16.1	12.2
Other acute URI	12.1	29.5	20.6	9.0	9.7	7.3	4.1
Influenza	41.2	53.6	59.4	43.1	45.2	28.0	14.0
Acute bronchitis	5.1	12.7	5.1	4.4	4.4	3.2	5.3
Pneumonia	2.0	*4.4	*2.1	*0.3	*1.4	2.3	*2.2
Other respiratory conditions	1.7	5.5	2.5	*1.1	*1.2	*0.7	*1.2

* The authors of this study indicate that these numbers do not meet standard of reliability or precision.

According to these numbers, children, particularly young children under five years of age, frequently experience respiratory illnesses associated with absence from daycare or school and eventually require visits at a health care provider. A similar declining number of respiratory infections per year with increasing age has been observed in the classical Tecumseh and Michigan studies conducted in

the late 1960s and early 1970s as reviewed elsewhere (Monto 2002). In a global survey presented by WHO, lower respiratory infections were listed, after ischemic heart disease and cerebrovascular disease, as the third leading cause of death in 2004, responsible for over 7 percent of all deaths http://www.who.int/healthinfo/global_burden_disease/2004_report_update/en/index.html. Furthermore, if the global burden of disease is expressed as "disability adjusted life years lost" (DALYs), lung infection ranked as number 1, before HIV/AIDS and neoplasms (Mizgerd 2006). In this report, the category "lung infections" includes "influenza and pneumonia" (ICD-10 codes J10 – J18) and "other acute lower respiratory tract infections" (ICD-10 codes J20 – J22), but excludes "tuberculosis" and "HIV infections resulting in infectious and parasitic diseases". A more recent study supports the concept that respiratory infections are important factors in global health and as the cause of life years lost (Lozano, Naghavi et al. 2012). From these statistics it is impossible to estimate in which proportions bacteria and viruses are involved. Ruuskanen and colleagues estimated that approximately 200 million cases of community-acquired pneumonia caused by viruses occur every year, half of those in children (Ruuskanen, Lahti et al. 2011). In adults, viruses are the predominant cause of the common cold (Makela, Puhakka et al. 1998). Using highly sensitive molecular methods for the detection of a large variety of respiratory viruses, 92 % of children with a recent onset of common cold were found virus positive (Ruohola, Waris et al. 2009). These reports underscore the fact that respiratory tract infections, including those caused by viruses, present a significant public health problem.

Understanding the global, regional, and local epidemiology of respiratory pathogens, identifying the spectrum of clinical symptoms caused by a given pathogen, adequate use of antibiotics and antiviral drugs, monitoring the possible development of drug-resistance, prevention of nosocomial spread, update of the seasonal influenza vaccine composition, and rapid identification and characterization of "novel" or unusual respiratory pathogens, are some of the reasons why surveillance of respiratory pathogens is conducted at the national and international level.

As one of its first surveillance programs, WHO established a global influenza surveillance network, currently named Global Influenza Surveillance and Response System (GISRS). At present, this network consists of 140 National Influenza Centers (NIC) in 110 WHO member states, 6 WHO Collaborating Centers (Atlanta, Beijing, London, Melbourne, Memphis, Tokyo) and 4 Essential Regulatory Laboratories (Hertfordshire, UK; Rockville, MD; Tokyo, Japan; Woden ACT, Australia). The GISRS plays a central role in the influenza vaccine virus selection process (Ampofo, Baylor et al. 2012). NICs collect clinical samples from patients with influenza-like illnesses (ILI) or acute respiratory infection (ARI), attempt to detect influenza and other respiratory viruses in these samples by virus culture or by other methods, performing typing, subtyping, and initial genetic and antigenic characterization of these viruses. NICs send selected virus isolates and/or clinical specimens to the regional WHO Collaborating Centers for further analyses. Based on results

from these analyses and on information on the geographical spread of these viruses and their impact, a group of experts under the co-ordination of WHO Headquarters makes recommendations as to what viruses should be included in influenza vaccines. Selected viruses are made available to Essential Regulatory Laboratories, which prepare high growth reassortant viruses that then are distributed to vaccine manufacturers. Since 1973 WHO has made formal recommendations for the vaccine composition (Klimov, Garten et al. 2012), until 1977 for influenza A(H3N2) and influenza B viruses, and since the re-emergence of influenza A(H1N1) viruses in 1977 (Kung, Jen et al. 1978) also a vaccine virus for this subtype has been included in the recommendations. Table 4 presents WHO recommended influenza vaccine strains for seasonal and pandemic vaccines. This list, spanning over 40 years, contains 10 different influenza A(H1N1) viruses, 24 influenza A(H3N2) viruses, and 15 influenza B viruses. Another important role of GISRS is to rapidly identify unusual subtypes of influenza A viruses from zoonotic transmissions. For the surveillance of animal influenza viruses, WHO is in close cooperation with the World Organization for Animal Health (OIE) and the UN Food and Agriculture Organization (FAO) (http://www.offlu.net/). A number of national and international surveillance systems have been established in addition to the WHO network (Fleming, van der Velden et al. 2003; Fleming and Elliot 2008). As an example, the European Influenza Surveillance Scheme (EISS) has started to compile surveillance data from 7 European countries in 1995 (Snacken, Manuguerra et al. 1998). In 2008, this surveillance network was integrated into the activities of the European Center for Disease prevention and Control (ECDC) in Stockholm. Influenza surveillance in Europe has shed light on influenza activity during a number of influenza seasons (Paget, Marquet et al. 2007). The Community Network of Reference Laboratories (CNRL) has provided laboratory data on influenza virus detections (Meijer, Valette et al. 2005; Meijer, Brown et al. 2006). This network also provides virological data on the occurrence of RSV in Europe (Meerhoff, Mosnier et al. 2009).

An important task of laboratory-based influenza surveillance is to test circulating influenza viruses for their susceptibility to antiviral drugs. When the adamantanes amantadine and rimantadine more widely came into clinical use, within a few years virtually all human influenza A viruses became resistant to these two compounds (Ziegler, Hemphill et al. 1999; Bright, Medina et al. 2005). In influenza A(H1N1) viruses circulating in Europe during the 2007 – 2008 season, resistance to the neuraminidase inhibitor oseltamivir emerged

Table 4. Influenza virus strains recommended by WHO to be included in seasonal influenza vaccines 1973 – 2013 for the Northern Hemisphere and for the pandemic vaccine 2009.

Season	Influenza A (H1N1)	Influenza A (H3N2)	Influenza B
1973/74		A/England/42/72[1]	
1974/75		**A/Port Chalmers/1/1973[2]**	**B/Hong Kong/05/1972**
1975/76		**A/Scotland/840/74**	B/Hong Kong/05/1972
1976/77		**A/Victoria/3/75**	B/Hong Kong/05/1972
1977/78		A/Victoria/3/75	B/Hong Kong/05/1972
1978/99	**A/USSR/90/77**	**A/Texas/1/77**	B/Hong Kong/05/1972
1979/80	A/USSR/90/77	A/Texas/1/77	B/Hong Kong/05/1972
1980/81	**A/Brazil/11/78**	**A/Bangkok/01/79**	**B/Singapore/222/79**
1981/82	A/Brazil/11/78	A/Bagkok/01/79	B/Singapore/222/79
1982/83	A/Brazil/11/78	A/Bangkok/01/79	B/Singapore/222/79
1983/84	A/Brazil/11/78	**A/Philippines/2/82**	B/Singapore/222/79
1984/85	A/Chile/1/83	A/Philippines/2/82	**B/USSR/100/83**
1985/86	A/Chile/1/83	A/Philippines/2/82	B/USSR/100/83
1986/87	A/Chile/1/83	**A/Christchurch/4/85**	**B/Ann Arbor/1/86**
1987/88	**A/Singapore/6/86**	**A/Leningrad/360/86**	B/Ann Arbor/1/86
1988/89	A/Singapore/6/86	**A/Sichuan/02/87**	**B/Beijing/1/87**
1989/90	A/Singapore/6/86	**A/Shanghai/11/87**	**B/Yamagata/16/88**
1990/91	A/Singapore/6/86	**A/Guizhou/54/89**	B/Yamagata/16/88
1991/92	A/Singapore/6/86	**A/Beijing/353/89**	B/Yamagata/16/88
1992/93	A/Singapore/6/86	A/Beijing/353/89	B/Yamagata/16/88
1993/94	A/Singapore/6/86	**A/Beijing/32/92**	**B/Panama/45/90**
1994/95	A/Singapore/6/86	**A/Shangdong/9/93**	B/Panama/45/90
1995/96	A/Singapore/6/86	**A/Johannesburg/33/94**	**B/Beijing/184/93**
1996/97	A/Singapore/6/86	**A/Wuhan/359/94**	B/Beijing/184/93
1997/98	**A/Bayern/7/95**	A/Wuhan/359/94	B/Beijing/184/93
1998/99	**A/Beijing/262/95**	**A/Sydney/5/97**	B/Beijing/184/93
1999/00	A/Beijing/262/95	A/Sydney/5/97	B/Beijing/184/93
2000/01	**A/New Caledonia/20/99**	**A/Moscow/10/99**	B/Beijing/184/93
2001/02	A/New Caledonia/20/99	A/Moscow/10/99	**B/Sichuan/379/99**
2002/03	A/New Caledonia/20/99	A/Moscow/10/99	**B/Hong Kong/330/2001**
2003/04	A/New Caledonia/20/99	A/Moscow/10/99	B/Hong Kong/330/2001
2004/05	A/New Caledonia/20/99	**A/Fujian/411/2002**	**B/Shanghai/361/2002**
2005/06	A/New Caledonia/20/99	**A/California/7/2004**	B/Shanghai/361/2002
2006/07	A/New Caledonia/20/99	**A/Wisconsin/67/2005**	**B/Malaysia/2506/2002**
2007/08	**A/Solomon Islands/3/2006**	A/Wisconsin/67/2005	B/Malaysia/2506/2002
2008/09	**A/Brisbane/59/2007**	**A/Brisbane/10/2007**	**B/Florida/4/2006**

2009	**A/California/7/2009**[3]		
2009/10	A/Brisbane/59/2007	A/Brisbane/10/2007	**B/Brisbane/60/2008**
2010/11	**A/California/7/2009**	**A/Perth/16/2009**	B/Brisbane/60/2008
2011/12	A/California/7/2009	A/Perth/16/2009	B/Brisbane/60/2008
2012/13	A/California/7/2009	A/Victoria/361/2011	**B/Wisconsin/1/2010**
2013/14	A/California/7/2009	A/Victoria/361/2011	**B/Massachusetts/2/2012**

[1]The A/England/42/72(H3N2) has been included in seasonal vaccines prior to 1973, but at that time, the recommendations have not yet been done by WHO.
[2]Virus strains in bold font present a change of virus strain in that season's vaccine.
[3]The vaccines produced for the 2009 pandemic contained only the A(H1N1)pdm09 virus.

(Lackenby, Hungnes et al. 2008; Meijer, Lackenby et al. 2009), and quickly spread around the globe (Moscona 2009) http://www.who.int/influenza/patient_care/antivirals/oseltamivir_summary_south_2008/en/.

Clinical diagnosis of respiratory infections. Based on the clinical picture alone it is rarely possible to specifically identify the causing agent of a respiratory infection. A large study aimed to identify the most predictive signs and symptoms for influenza in patients with influenza-like illness (Monto, Gravenstein et al. 2000). Patients presenting with cough and fever were most likely to experience a laboratory-confirmed influenza infection with a positive predictive value of 79 %. In another study, patients with influenza-like symptoms were recruited to determine the efficacy of treatment with zanamivir (Makela, Pauksens et al. 2000). In this investigation, 277 of 356 patients (78 %) were found influenza-positive by laboratory testing. Both studies have been conducted with adolescent or adult, otherwise healthy individuals. In a study of 683 hospitalized children with laboratory-confirmed influenza A or B infection it was shown that the clinical presentation in young children is less specific than in older children and adults, and therefore laboratory diagnosis is often justified (Peltola, Ziegler et al. 2003).

2.4 Laboratory diagnosis of respiratory tract infections

2.4.1 Patients and specimens

Over the course of respiratory tract infections the amount of pathogen present in respiratory tract secretions diminishes. Viruses can be present before clinical symptoms occur. During the first days of the acute illness the amount of virus reaches its maximum and thereafter diminishes over the course of a few days. For that reason it is important to collect samples for the detection of viruses by culture, by antigen detection, or by detection of nucleic acids early during the clinical illness (Ronkko, Ikonen et al. 2011). For serodiagnosis of respiratory infection, the first serum sample should be drawn early during the acute phase and the convalescent phase sample 2 to 4 weeks later.

Specimens suitable for the detection of respiratory tract pathogens include nasopharyngeal aspirates, nasopharyngeal and/or throat swabs (Gnarpe, Lundback et al. 1997; Heikkinen, Salmi et al. 2001; Waring, Halse et al. 2001; Meerhoff, Houben et al. 2010) nasopharyngeal washes (Berg, Yolken et al. 1980), sputum (van Kuppeveld, Johansson et al. 1994), and, in special cases, specimens that require more invasive sampling methods such as middle ear fluids, tracheal aspirates, pleural fluid specimens, bronchoalveolar lavage specimens, transthoracic needle aspirates, and autopsy specimens. Although not a very pleasant method, nasopharyngeal aspirates have proven to be an excellent clinical sample for the detection of respiratory viruses by several methods including antigen detection by immunofluorescent antibody staining or by immunoassays, by culture, and by molecular methods. Nasopharyngeal aspirates appear to contain large numbers of exfoliated, epithelial cells, some of which may contain virus antigens. During recent years, a flocked swab has found wide application. This swab is manufactured such that it traps epithelial cells between the brushes and efficiently releases them into either a transport medium or an extraction buffer (Abu-Diab, Azzeh et al. 2008; Faden 2010; Ampofo, Baylor et al. 2012).

2.4.2 Development of diagnostic methods

Based on patient’s clinical symptoms, it is difficult or impossible to identify the causing agent because infections with many different respiratory pathogens can present with the same symptoms. The traditional methods for the diagnosis of respiratory virus infections are virus culture and serology performed on acute and convalescent-phase sera (Monto and Kioumehr 1975). With these methods, only in rare instances was it possible to obtain a result as long as it would have been of any benefit for the patient or for the treating physician. When the antiviral drug amantadine came in clinical use, and particularly when the risk of nosocomial infections in the hospital environment was better understood, the need for more rapid diagnostic methods became evident.

Four main categories of methods are available for diagnosing infections caused by respiratory viruses and *Mycoplasma pneumoniae*. These include viral and bacterial culture, detection of microbial antigens with immunological methods, amplification of nucleic acids, and serology.

2.4.2.1 Antigen detection methods

Time-resolved fluoroimmunoassay (TR-FIA). The TR-FIA technology was developed in the 80’ies in the Wallac Research Laboratories (Turku, Finland). The principles of the technology were described by Soini and Kojola 1983 (Soini and Kojola 1983) and Hemmilä et al. (Hemmila, Dakubu et al. 1984). This method allows for the specific identification of proteins with high sensitivity and within a short time. This method was soon adapted for the rapid detection of common respiratory viruses in clinical samples obtained from patients with upper and lower

respiratory tract infections (Halonen, Meurman et al. 1983). Specimen preparation before testing is simple, i.e., it does not require isolation of infected cells from nasopharyngeal secretions as necessary for fluorescent antibody staining. Usually the sample is eluted or diluted in a detergent-containing buffer and then homogenized by ultrasonic treatment.

The method for detection of influenza infections by TR-FIA using monoclonal antibodies as immunoreagents was first published by Walls and colleagues (Walls, Johansson et al. 1986). One year later Hierholzer and colleagues described a similar, slightly refined TR-FIA method for the detection of adenovirus antigens in respiratory secretions and in stool specimens (Hierholzer, Johansson et al. 1987). This so-called ”one-incubation method” was then also adapted for the detection of influenza A and B viruses (Nikkari, Halonen et al. 1989), of RSV (Waris, Halonen et al. 1988), and of parainfluenza virus, types 1 – 3 (Hierholzer, Bingham et al. 1989). In some of these investigations, the sensitivity of TR-FIA methods was comparable to that of previously used EIA and RIA tests using polyclonal antisera as immunoreagents. TR-FIA was found to be more sensitive and also more specific than the older immunoassays. A thorough, systematic comparison against virus culture, which was at that time regarded as the ”golden standard”, has never been performed. However, TR-FIA with one short incubation time proved to be a useful assay for the simultaneous detection of several viruses and is well suited for routine diagnosis of respiratory viral infections in a diagnostic laboratory. Equivocal results can be confirmed by a control test adding an incubation step with the unlabeled virus-specific antibody before adding the Eu-conjugated antibody.

This TR-FIA method for the detection of seven common respiratory viruses was used in several clinical and epidemiological studies (Ruuskanen, Arola et al. 1989; Arola, Ziegler et al. 1990; Mertsola, Ziegler et al. 1991; Waris, Meurman et al. 1992; Makela, Puhakka et al. 1998).

In order to make this technique more widely available to laboratories without TR-FIA equipment, Scalia et al. (Scalia, Halonen et al. 1995) biotinylated the same monoclonal antibodies as used in TR-FIA studies. Biotin labeling allowed the use of peroxidase-labeled streptavidin conjugates and widely available EIA spectrophotometers. In that study, the EIA format turned out to be less sensitive than the corresponding TR-FIA method.

Methods for virus antigen detection still are widely used in diagnostic laboratories, but during recent years they have to some extent been replaced by PCR.

Immunofluorescent antibody staining (IF). The first practical technique that became available for the rapid diagnosis of respiratory tract infections was fluorescent antibody staining of virus-infected epithelial cells. This technique was developed and refined in the 1960s. Several technical problems had to be overcome in order to make this method reliable for laboratory diagnosis. One of the major problems was the specificity of the immunoreagents. Initially high-titered human sera were used, later animal hyperimmune sera, and nowadays mouse monoclonal anti-

bodies are the standard. Additional problems were the specimen transport and preparation. Several methods for the purification of virus-infected, exfoliated epithelial cells have been described to free the cells from the contaminating mucus (Ukkonen and Julkunen 1987). Combining these purification methods with highly specific, commercially available monoclonal antibodies significantly improved the reliability of this technique and it is still used in many diagnostic laboratories (Shen, Zhaori et al. 1996). Reading of samples can be influenced by non-specific staining, and the quality of the test result always depends on the interpretation of the microscopist. On the other hand, an experienced microscopist may find a single infected cell on a stained slide, which renders this technique highly sensitive.

IF techniques have been used lately alongside with molecular methods in the search for the emerging viruses of this century (Yuen, Chan et al. 1998; van der Hoek, Pyrc et al. 2004; Zaki, van Boheemen et al. 2012).

Solid-phase immunoassays. The direct detection of viral antigens in clinical specimens has first been accomplished for the Australia antigen of hepatitis B virus using radioimmunoassay technology (Walsh, Yalow et al. 1970). RIA has been widely applied in the laboratory diagnosis of respiratory virus infections (Sarkkinen, Halonen et al. 1981). The availability of enzyme-labeled conjugates has simplified the further development of solid-phase immunoassays for laboratory diagnostic purposes (Berg, Yolken et al. 1980). RIA and EIA have been found to be of equal sensitivity. A comparative study of the sensitivity of RIA and EIA for the detection of RSV, parainfluenza virus type 2, and adenovirus in NPAs of children showed that both methods were able to detect between 1 and 30 ng of virus protein per ml (Sarkkinen, Halonen et al. 1981). In another study, RIA was used for direct detection of viral antigens in nasopharyngeal secretions and results were compared with serology carried out by EIA (Meurman, Sarkkinen et al. 1984). Large numbers of specimens can be processed using RIA or EIA. With the numeric output of results by RIA, EIA, and other similar immunoassays, subjective interpretation of results as often observed by IF could be excluded.

Rapid point-of-care tests can be completed within 15 – 30 minutes without any special equipment and can therefore be performed in a doctor's office, in an outpatient clinic or a hospital ward. These so called "point-of-care" tests have been in clinical use for over 20 years for the detection of influenza viruses and RSV (Rothbarth, Hermus et al. 1991; Waner, Todd et al. 1991; Ryan-Poirier, Katz et al. 1992). The diagnostic sensitivity and reliability of such rapid tests remains to be a topic of discussion (MMWR 2012) (www.cdc.gov/mmwr/pdf/wk/mm6143.pdf). The further development and refinement of the TR-FIA method lead to the invention of a two-photon excitation fluorometry technique. This method has been successfully applied for the detection of respiratory pathogens in clinical specimens (Koskinen, Vainionpaa et al. 2007). It is highly specific and sensitive, and positive results can be obtained within a few minutes. However, this method requires sophisticated equipment.

2.4.2.2 Virus culture

Traditional cell culture. Many human respiratory viruses can be propagated in cell cultures or in hen's eggs. Virus culture continues to play an important role, because it provides an infectious virus for further characterization and thus is complementary to rapid and molecular methods. Virus culture is also important in the identification of previously unknown viruses, such as hMPV (van den Hoogen, de Jong et al. 2001), SARS coronavirus (Peiris, Yuen et al. 2003), and MERS-coronavirus (Zaki, van Boheemen et al. 2012). When presumably dealing with previously unknown viruses, a variety of cell lines must be inoculated with a clinical specimen in order to increase chances that the virus will grow at least in one of these cell lines (van der Hoek, Pyrc et al. 2004; Zaki, van Boheemen et al. 2012).

In many respiratory tract infections, patients shed virus only during a short period, sometimes already before the onset of symptoms, and usually ceases within a few days. Specimens for virus culture must therefore be collected at an early stage of the illness. It often takes time to get the virus adapted to grow in a certain cell line and eventually one or more blind passages need to be performed to obtain an isolate. Virus type and subtype can be identified by immunological methods using hyperimmune sera or monoclonal antibodies (Pyhala and Pyhala 1987; Harmon 1992) or by molecular methods.

Rapid culture of viruses. Rapid culture of viruses allows for the identification of a virus in cell culture using immunofluorescent or immunoperoxidase staining methods before a virus-induced cytopathic effect can be microscopically identified. Rapid culture is commonly performed in shell vials or in multi-cluster dishes (Espy, Smith et al. 1986; Espy, Hierholzer et al. 1987; Trabelsi, Pozzetto et al. 1992). As early as 18 hours after inoculation, virus replication in infected cells can be visualized after immunostaining (McQuillin, Madeley et al. 1985; Routledge, McQuillin et al. 1985; Waris, Ziegler et al. 1990; Ziegler, Hall et al. 1995). The sensitivity of rapid culture methods is comparable to that of standard virus culture, and the result is often available in due time to be helpful in the management of the patient.

2.4.2.3 Serology

Complement fixation test (CFT). The principles of complement fixation test (CFT) were adapted from Hawkes (Hawkes 1979), and Casey (Casey 1965). Most reliable results are obtained when acute- and convalescent-phase sera are tested simultaneously with a demonstration of a significant titer rise. In certain cases, a high titer obtained from a single serum by CFT may be indicative of recent infection. Optimal timing and interval of serum collection has been studied for influenza by Pyhälä and Kleemola (Pyhala and Kleemola 1976) and was determined to be between 3 and 4 weeks.

Hemagglutination inhibition (HI test). The standard HI test can be used for viruses that can agglutinate erythrocytes (Hierholzer, Suggs et al. 1969). HI tests are widely used for the serodiagnosis of influenza infections and for the monitoring of

the efficacy of influenza vaccinations (Pyhala, Kleemola et al. 1985; Pyhala, Ikonen et al. 2001; Strengell, Ikonen et al. 2011). Antibodies against the viral hemagglutinin HA, measured by the HI test, presumably protect from influenza infection with a homologous virus (Kojimahara, Maeda et al. 2006).

Neutralization test. Neutralization has been widely used for typing of viruses but can be used for the measurement of specific antibodies as well (Ludwig, Brundage et al. 1998; Lehtoranta, Villberg et al. 2009).

Solid-phase enzyme immunoassays (EIA). The specificity of solid-phase immunoassys depends largely on the specificity of the antigens used (Engvall, Jonsson et al. 1971; Engvall and Perlmann 1971; Van Weemen and Schuurs 1971; McIntosh, Wilfert et al. 1978; Voller, Bartlett et al. 1978). There are studies showing that EIA detected considerably more diagnostic titer rises than CFT between acute and convalescent sera when antibodies against influenza A and B viruses, parainfluenza viruses, and adenoviruses were measured by both methods and the results were compared with each other (Meurman, Ruuskanen et al. 1984; Julkunen, Pyhala et al. 1985).

Antibodies of the IgM class are normally produced in an immunocompetent individual during the first contact with a certain pathogen. In terms of respiratory viruses, this usually happens in children and young adults who have not earlier been infected with the specific or a closely related pathogen. Assays for the detection of IgM-class antibodies can usually be performed with a single serum sample taken early during the course of an infection (Hirschberg, Krook et al. 1988; Vikerfors, Grandien et al. 1988).

Among adolescents, adults, and the elderly with respiratory infections, the appearence of IgM antibodies is inconsistent, partly because the majority of these infections are re-infections (Makela, Nikkari et al. 1995). In contrast, also in reinfections, IgG class antibodies usually rise significantly, and a four-fold increase in antibody titers between acute- and convalescent-phase sera can in many cases be demonstrated as proof of recent infection.

Distinction between acute infection and pre-existing immunity by avidity testing of antibodies. The presence of IgG class antibodies in a single serum sample does not reveal whether these antibodies are indicative of an acute or very recent infection or whether they are derived from an infection with the same pathogen in the past. After primary infection with a pathogen, the avidity of pathogen-specific IgG class antibodies maturates over a period of weeks or months. With special methods, the degree of an antibody's avidity can be identified and thus an acute infection can be differentiated from an infection in the past. This method has been developed in the late 1980s for differentiation between acute and re-infection with rubella virus (Hedman and Seppala 1988; Rousseau and Hedman 1988) and has since been applied for many different pathogens. (Smolander, Koskinen et al. 2010; Freitas, Silva et al. 2011).

2.4.2.4 Molecular methods

The availability of a thermostable DNA polymerase, i.e., the Taq-polymerase opened the way for the polymerase chain reaction (PCR) method into the diagnostic laboratory in the late 1980s. Saiki and colleagues (Saiki, Gelfand et al. 1988) first published the PCR format that basically still forms the principle for all the refined methods that have been developed and introduced thereafter. In virology, the method was first applied for the detection of DNA viruses, but soon the method could also be applied for RNA viruses when high quality reverse transcriptase enzymes became available commercially.

Isolation of *M. pneumoniae* requires a complex growth medium and it may take several weeks to obtain a positive result. Therefore, PCR greatly improved the possibility to detect this pathogen in clinical specimens, and it was one of the first microbes that could be detected by PCR (Bernet, Garret et al. 1989; Jensen, Sondergard-Andersen et al. 1989). The first publications on adenovirus PCR appeared in 1990 (Allard, Girones et al. 1990). The PCR technique and its modifications helped in the identification of viruses for which no susceptible cell lines or other detection systems are available. Picornaviruses were the first RNA viruses that could be detected and identified by RT-PCR (Hyypia, Auvinen et al. 1989). PCR was also applied for the detection of pathogens for which no susceptible cell lines or other isolation systems are available. An illustrative example are the bocaviruses (Allander, Tammi et al. 2005). Also for viruses that can be grown in cell cultures, PCR was found to be a considerably more sensitive method. In a careful comparison of rhinovirus culture in HeLa Ohio cells and RT-PCR, Mäkelä and colleagues detected 103 rhinovirus-positive specimens by PCR but only 80 by culture (Makela, Puhakka et al. 1998). Due to their antigenic diversity, for certain virus families it would also be difficult to prepare suitable reagents to be used in immunoassays (Wong, Pabbaraju et al. 2009) and therefore PCR is a practical method for the detection of such viruses.

Bioinformatics assists in the identification of conserved gene sequences among viruses belonging to the same family. Using this information it was possible to develop PCR methods that widely detect members of rhinoviruses (Nokso-Koivisto, Raty et al. 2004), of the paramyxovirus family (van Boheemen, Bestebroer et al. 2012), or coronaviruses (Moes, Vijgen et al. 2005). This pancoronavirus PCR greatly assisted in the recent identification of the MERS-coronavirus in summer 2012 (Zaki, van Boheemen et al. 2012).

An important improvement of the PCR technology is the possibility to combine reagents for the detection of different pathogens in one reaction, i.e., multiplex PCR. This development considerably contributed to the wider availability of the technology in diagnostic laboratories because multiplex PCR helps saving costs for reagents. Multiplex PCR methods have been widely applied for the detection of respiratory viruses in clinical samples (Templeton, Scheltinga et al. 2004; Ronkko, Ikonen et al. 2011). Assays for the detection of up to 16 different respiratory pathogens are nowadays available from commercial sources.

As with any laboratory technique, also for PCR methods the quality of the clinical specimen is of utmost importance. Viruses and their nucleic acids start disappearing from respiratory secretions only a few days after onset of diseases, often already during the acute phase of the illness (Ronkko, Ikonen et al. 2011). However, if respiratory samples are collected from children presenting early with symptoms of the common cold, with the aid of PCR technology an etiologic agent can be identified for almost all patients (Ruohola, Waris et al. 2009). Using highly sensitive methods, it is not unusual to detect more than one pathogen in a clinical specimen. Also in the study by Ruohola and colleagues, 25 percent of the study patients harbored two, three, or even four respiratory viruses simultaneously. In these cases, it is difficult to determine which of the viruses is the true causing agent. Quantitative PCR and/or serological methods may help in finding answers to this question. Based on results from quantitative PCR, the viruses showing the lowest Ct value might be the one that is acutely replicating and thus causing the symptoms. A diagnostic titer rise or the presence of virus-specific IgM antibodies may further help determining which of the viruses causes the current illness (Don, Soderlund-Venermo et al. 2011).

Although PCR is a highly sensitive, flexible and powerful diagnostic method, for the time being it does not completely replace virus culture and other commonly used diagnostic methods. Virus isolates are still required for the selection of vaccine viruses and for the determination of antiviral drug susceptibility.

3 Aims of the Study

The aim of the present thesis work was to develop and validate laboratory methods for the detection of seven common respiratory viruses and of *Mycoplasma pneumoniae* and to apply these methods on clinical specimens collected for different purposes.

The specific aims were:

1. To determine the diagnostic sensitivities and specificities of virus culture techniques, antigen detection by TR-FIA, gene amplification by PCR and CFT serology for the identification of adenovirus infections in military recruits
2. To clarify the role of *M. pneumoniae* in acute otitis media.
3. To evaluate the best suited type of clinical specimens for the detection of *M. pneumoniae*
4. To demonstrate the value of serology in the diagnosis of infections caused by seven common respiratory viruses and *M. pneumoniae.*
5. To study the epidemiology of adenoviruses, influenza A and B viruses, parainfluenza viruses type 1, 2, and 3, RSV and *M. pneumoniae* based on clinical specimens collected from patients with acute respiratory infections representing several population groups over a period of 16 years from 1989 to 2005.

4 Patients, Clinical Specimens, and Laboratory Methods

Respiratory specimens and paired serum samples have been collected from individuals presenting with symptoms of an ongoing respiratory infection. Participating doctors and medical centers were requested to take respiratory and acute-phase serum samples as early as possible during the course of illness, preferably within 1 – 3 days. Convalescent-phase sera were usually taken 2 – 4 weeks later. Testing of clinical specimens was provided to the treating physician as a primary diagnostic service. Specimens were collected in order to aid clinicians in their decisions on treatment of the patient or on the design of counter-measures to limit the epidemic, e.g., in military training centers. The number of patients and specimens collected, as well as the methods used in each study, is presented in Table 5.

Table 5. Patient groups included in these studies, and specimens collected

Publication	Patients	Number of infection episodes	Type of specimens collected	Methods used
I	Military recruits with ARI	269	NPA, acute and convalescent sera	TR-FIA, PCR, culture, CFT
II	Children with AOM	138	NPA, MEF, acute and convalescent sera	TR-FIA, PCR, CFT EIA
III	Children with AOM	447	NPA, MEF, acute and convalescent sera	TR-FIA, CFT, EIA
IV	Military recruits with X-ray confirmed pneumonia	32	NPA, TS, sputum, acute and convalescent sera	PCR, CFT
This thesis	Civil and military patients of all age groups with ARI	12,020	NPA, acute and convalescent sera	TR-FIA, CFT

4.1 Evaluation of laboratory methods for the detection of adenoviruses (I)

During an adenovirus outbreak in military training centers between January and April, 1991, clinical specimens were collected from 269 patients. NPA specimens were studied by rapid and conventional culture in A-549 cells, by PCR, by EIA, and by TR-FIA. Acute and convalescent phase serum specimens were tested by adenovirus CFT using an in-house produced antigen prepared from adenovirus type 1 infected, cultured cells.

4.2 The role of *M. pneumoniae* as a causative agent of AOM in children (II)

The FinOM Cohort Study was conducted from 1994 to 1997 and included 329 children with AOM. From 138 children 380 MEF specimens were tested by a PCR method specific for the P1 protein gene of *M. pneumoniae*. Corresponding NPA specimens obtained during the same episode were tested by PCR for the presence of *M. pneumoniae*. In 268 episodes, an NPA was collected simultaneously with the MEF. For 225 episodes, paired acute and convalescent phase serum specimens were tested by CFT as described (I).

4.3 Evaluation of optimal specimens for the detection of *M. pneumoniae* by PCR and by CFT (III)

Between October 2001 and January 2002, NPA, sputum, throat swab, and acute and paired serum specimens were collected from 32 military recruits aged 22 – 29, hospitalized with an X-ray confirmed pneumonia. Detection of *M. pneumoniae* DNA was done by PCR. *M. pneumoniae* specific antibodies were measured by CFT using an in-house prepared antigen extracted from cultured *M. pneumoniae* bacteria (strain ATCC 29342).

4.4 Comparison of serology and antigen detection in the diagnosis of AOM in children (IV)

From 447 episodes experienced by 179 children 2 months to 2 years of age, a complete set of clinical specimens (NPA, MEF, and acute and convalescent sera) was available. NPA and MEF specimens were tested for the presence of adenovirus, influenza virus types A and B, parainfluenza virus types 1, 2, and 3, and RSV by TR-FIA. From acute and convalescent phase sera, antibodies to RSV were measured by EIA using a commercial antigen. Antibodies to adenovirus, influenza virus types A and B, parainfluenza virus types 1, 2, and 3 were assessed by CFT.

4.5 Sixteen years laboratory surveillance of seven common respiratory viruses and *M. pneumoniae* in Finland (this thesis)

Between September 15, 1989, and June 15, 2005, 12,020 events of acute respiratory tract infections (ARI) or influenza-like illnesses (ILI) were analyzed. Specimens were collected from different population groups. The types of specimens collected were NPA and paired acute and convalescent phase sera. Both types of specimens were available only from a fraction of the entire population. The numbers of specimens are shown in Table 6.

Table 6. Origin and number of clinical specimens in the 16-year laboratory surveillance of respiratory tract infections

	Number of cases with			
Population group	NPA only	Paired blood specimens only	NPA and paired blood specimens	Total
Military recruits and personnel	1594	2606	2981	7181
Children ≤17 years of age	2833	12	874	3719
Health care workers	178	78	223	479
Municipal health care center patients	586	3	5	594
Insufficient information available	33	15	9	47
Total	5214	2714	4092	12020

Patients were diagnosed and treated at the former Central Military Hospital, Helsinki, Finland, at health care centers of different garrisons of the Finnish Defense Forces, state and private health care centers, at the pediatric department of the North Karelian Central Hospital in Joensuu, Finland, and the Aurora Hospital infectious disease clinic, Helsinki, Finland. The age of the patients ranged from 0 to 96 years.

Respiratory specimens were collected during the acute phase of the illness, usually within three days after the onset of symptoms. For identification of respiratory

pathogens, an NPA specimen was collected from both nostrils by a mucus extractor (Uno Plast A/S, Hundestad, Denmark). For that purpose, the catheter was inserted into the nostril (3 – 6 cm, depending on the age of the patient) before the vacuum was applied. The catheter was left in place for a few seconds in order to stimulate mucus secretion by the airflow, before it was slowly withdrawn. This procedure was repeated with the other nostril using the same catheter. Typically, 0.5 to 2.0 ml of mucus was obtained. Mucus remaining in the catheter was collected by flushing the tube with 1 – 2 ml of physiological saline solution. For serological diagnosis, acute and convalescent phase serum specimens were collected. The first serum sample was usually collected simultaneously with the NPA specimen, and the convalescent phase serum was taken 2 – 4 weeks later. NPA specimens were tested immediately after arrival in the laboratory. Remaining NPA material was stored frozen at -70°C. Serum specimens were stored refrigerated at 4ºC -8ºC or at -20ºC until tested. Each specimen was sent with a standardized referral form containing adequate patient information and data on clinical findings.

4.5.1 Laboratory methods used in the 16-year surveillance

NPA specimens were tested by TR-FIA for the presence of influenza A and influenza B, parainfluenza viruses type 1, 2, and 3, RSV and adenovirus antigens (Halonen, Herholzer et al. 1996). From acute and convalescent serum specimens, antibodies against the same viruses and against *M. pneumoniae* were measured by CFT (Hawkes 1979).

Clinical information and laboratory results were compiled in a Microsoft Access-based in-house refined database. Results from antigen detection by TR-FIA and from serology by CFT were compared with each other and the occurrence of respiratory pathogens during the observation period as determined by both methods was recorded.

5 Results

Results from the studies in the separate publications are presented only briefly here. The major part of this Results section is devoted to the 16-year surveillance study.

5.1 Laboratory diagnosis of adenovirus infections (I)

A total of 238 NPA specimens from military conscripts with an acute respiratory tract infection have been studied by conventional virus culture, rapid virus culture, PCR, EIA, and TR-FIA. With these five methods combined, adenovirus infections were detected in 157 (66 %) of these 238 specimens. PCR and conventional virus culture with IF confirmation proved to be the most sensitive methods, yielding 147 and 146 positive results, respectively. Rapid culture and antigen detection by EIA and by TR-FIA were less sensitive. From 133 patients acute and convalescent phase serum specimens were available for testing by CFT. In 69 of these serum pairs, a diagnostic increase of adenovirus-specific antibodies could be measured. On the other hand, 22 patients were found adenovirus-positive by PCR and 17 by antigen detection methods but failed to mount a significant titre increase by CFT.

5.2 *Mycoplasma pneumoniae* in the nasopharynx and in the middle ear fluid of children with acute otitis media (II)

Of the 380 MEF specimens, 16 were found positive for *M. pneumoniae* by PCR. From seven of these 16 patients, an NPA was also available for testing, of which only one gave a positive result by PCR. Serological findings did not match well with PCR findings. In 225 serum pairs only 4 significant titer increases were noted by EIA or by CFT. Of the 16 children with a *M. pneumoniae* in the MEF as determined by PCR, none had a ≥4-fold titer increase. However, in four of these 16 serum pairs a significant titer decrease was noted. *S. pneumoniae, H. influenzae* and *M. catarrhalis* were found in some specimens. One specimen was positive for parainfluenza virus type 3 and two for rhinovirus.

5.3 Sputum proved to be the optimal specimen for the detection of *M. pneumoniae* by PCR (III)

For 22 of 32 patients with an X-ray confirmed pneumonia, a diagnosis of *M. pneumoniae* could be established by PCR. From all these 22 patients, the sputum specimen harbored the pathogen. NPA and throat swabs proved to be less optimal, as with those specimens, 6 and 10 cases, respectively, would have remained undiagnosed. From 15 patients CFT provided a significant titer increase between acute and

convalescent phase sera. In nine serum pairs, the titers remained on a diagnostically high level. Sera were not available from 4 patients. (Note: one patient is missing in table 1 of separate publication III, page 289. Sputum, NPA, and throat swab specimen of this patient were negative by PCR. His *M. pneumoniae* antibody titer in both acute and convalescent serum was 256).

5.4 Serological methods enhance the diagnostic accuracy in otitis media patients (IV)

In 447 AOM events NPA and MEF specimens were collected during the acute phase. In addition, paired acute and convalescent phase sera were available for testing. For 163 events a viral etiology could be established combining TR-FIA for antigen detection, EIA for measuring antibodies to RSV, and CFT for six other common respiratory viruses. Thirty-four events yielded a virus finding by antigen detection and by serological methods. Serology alone yielded 81 and TR-FIA alone 48 positive results, respectively. RSV, adenovirus, and parainfluenza virus type 3 were the most frequently identified viral pathogens.

5.5 The occurrence of seven common respiratory viruses and *M. pneumoniae*

Over a period of almost 16 years 12,020 cases of acute respiratory infection were investigated by TR-FIA antigen detection and/or CFT serology (Table 7). One or more respiratory pathogens were identified in 5,343 (44.5 %) cases. NPA samples were available from 9,306 patients. Of those, 9,200 were tested for the presence of influenza A and B, respiratory syncytial, parainfluenza virus types 1, 2 and 3, and adenovirus antigens by TR-FIA. In 106 specimens the sample volume was insufficient to allow testing for all seven viruses, and thus the tests performed were selected based on the prevailing epidemic situation. TR-FIA revealed 3,051 (32.8 %) virus-positive cases including 35 cases (0.38 %) with dual viral findings (Table 7). Paired serum specimens were obtained from 6,806 patients. Almost all of these serum pairs have been tested by CFT for the presence of antibodies against the seven viruses and against *M. pneumoniae*. A four-fold or greater rise in antibodies against one or more antigens was detected in 3,431 (50.4 %) cases (Table 7).

5.5.1 Seasonal variation of respiratory virus and *M. pneumoniae* infections

The monthly number of specimens tested varied greatly over the 16-year observation period. The highest number of samples were received during the winter months, especially when there was a significant epidemic caused by influenza viruses or by RSV (Figure 1 and Figure 2). Figure 1 presents the monthly numbers of positive findings by TR-FIA, while serological observations with the number of diagnostic antibody rises

to different viruses and *M. pneumoniae* are presented in Figure 2. It should be noted that CFT data was no longer available in 2005. Adenoviruses were identified every year with peak epidemic activity mainly between December and April. Influenza epidemics were also observed every year and the majority of positive influenza findings were observed between December and February or January and March depending on the year. Major influenza A virus epidemics were usually associated with the emergence of a new antigenic variant of the H3N2 subtype as was the case in the years 1998, 2002, and 2003. Exceptionally high numbers of influenza B virus infections were observed during the epidemic season 2002/2003, a season when influenza A virus was almost absent. During seven winter seasons (years 1990, 1992, 1994, 1995, 1998, 2004 and 2005), very low or no influenza B virus activity was noted (Figure 1 and Figure 2). Parainfluenza viruses were found only sporadically and the findings were more scattered throughout the year as compared to influenza viruses. Most of the parainfluenza virus infections were caused by type 3 according to the results obtained by TR-FIA. Sporadic cases of RSV were observed during any month of the year. However, major epidemic activity varied considerably from one year to another and epidemic peaks were found in winter months and in spring as well as in late fall (Figure 1 and Figure 2). In addition to viruses, the epidemic activity of *M. pneumoniae* infections was monitored by serology and several outbreaks were identified during the 16-year surveillance period. These outbreaks often stretched over several months during fall, winter, and spring, and significant epidemics were observed in 1991-1993, 1995-1996, 2000 and 2001 (Figure 2).

5.5.2 Distribution of respiratory infections in different age groups

To more systematically analyze the age-related morbidity caused by respiratory microbial pathogens, patients were classified in three age groups, children and adolescents ≤17 years of age, young adults from 18 to 30 years, and adults >30 years of age. Patients younger than 18 years of age represented 31 % of the study population, those between 18 and 30 years 63 %, and individuals older than 30 years 6 %, respectively (Table 6). Children under 5 years of age represented about 75% in the age group of children and adolescents. From 47 patients no information about their age was available. In these age groups, the prevalence of different respiratory pathogens varied considerably. In individuals <17 years of age, RSV, influenza A, and adenoviruses were in that order the most common pathogens. Individuals between 18 and 30 years of age showed a high incidence of adeno- and influenza A virus infections, which together accounted for 75 % of all viral findings in this age group. In individuals over 30 years of age, influenza A and B viruses were clearly the most predominant pathogens, while parainfluenza viruses, RSV and *M. pneumoniae* were less prominent.

5.5.3 Comparison of the diagnostic value of TR-FIA and CFT

Next, the diagnostic power of viral antigen detection from NPA specimens by TR-FIA and diagnostic antibody rises in paired serum specimens by CFT were compared. In 4,092 respiratory episodes it was possible to obtain a complete set of clinical samples, i.e., NPA and paired acute and convalescent phase sera, and most of these sets have been tested for all seven respiratory viruses (Table 8). A total of 2,682 virus positive findings were identified by either TR-FIA or by CFT, and 1,115 virus infections were diagnosed by both methods. A total of 343 virus findings were established by TR-FIA alone without a significant by CFT. On the other hand, 1,221 virus findings by CFT could not be verified by TR-FIA (Table 8).

5.5.4 Co-infections with multiple respiratory viral pathogens and *M. pneumoniae*

In 392 cases (7.3 % of all cases with a diagnostic finding) two pathogens were identified for the same infection episode, in 334 cases by the serological test (Table 9). In 58 cases double infections were identified by both methods or only by TR-FIA (data not shown). Co-infections occurred in many combinations of the different pathogens and thus the frequency of specific viral pathogens seemed to follow a pattern, which reflected the overall frequency of certain viral infections. Thus co-infections where adenoviruses or influenza A viruses were associated with each other or with another respiratory pathogen were most commonly seen. Three pathogens were identified simultaneously in 20 patients (Table 10) and, similar to double infections, adenoviruses and influenza A viruses were most commonly involved.

Table 7. Number of acute respiratory infection cases investigated for the presence of each pathogen and positive results obtained by TR-FIA and/or CFT during the 16-year period of observation from 1989 to 2005.

Pathogen	Cases investigated	Number (%) of cases positive by TR-FIA and/or by CFT	Cases investigated by antigen detection	Cases (%) positive by antigen detection	Cases investigated by serology	Cases (%) positive by serology
ADE	12020	1914 (15.9)	9306	777 (8.3)	6806	1518 (22.3)
INA	11951	1879 (15.7)	9206	1281 (13.9)	6805	1165 (17.1)
INB	12019	518 (4.3)	9305	315 (3.4)	6806	324 (4.8)
PIV1	12018		9305	23	6804	
PIV2	11957		9200	27	6796	
PIV3	12012		9300	109	6798	
PIV	12019	348 (2.9)	9305	159 (1.7)	6806	206 (3.0)
RSV	12015	666 (5.5)	9300	555 (6.0)	6799	143 (2.1)
MPn					6806	452 (6.6)

Abbreviations in this and the following tables:
Adeno = ADE, Influenza A = INA, Influenza B = INB, Parainfluenza viruses = PIV, Respiratory syncytial virus = RSV, *Mycoplasma pneumoniae* = MPn
Not done = ND

Table 8. Diagnostic findings in acute respiratory tract infection patients from whom both nasopharyngeal aspirate and paired serum specimens were available during the 16-year observation period from 1989 to 2005.

Virus	Total number of cases	Cases (%) positive by antigen detection or by serology or by both tests	Cases (%) positive by antigen detection and by serology	Cases (%) positive by antigen detection only	Cases (%) positive by serology only
ADE	4092	1062 (26.0)	381 (9.3)	61 (1.5)	620 (15.2)
INA	4067	988 (24.3)	565 (13.9)	110 (2.6)	313 (7.7)
INB	4091	257 (6.3)	122 (3.0)	29 (0.6)	106 (2.6)
PIV1	4091		4 (0.1)	4 (0.1)	32 (0.8)
PIV2	4039		3 (0.1)	6 (0.2)	23 (0.6)
PIV3	4086		10 (0.2)	18 (0.2)	60 (1.5)
PIV	4091	163 (4.0)	17 (0.4)	28 (0.7)	115 (2.8)
RSV	4080	212 (5.2)	30 (0.7)	115 (2.8)	67 (1.6)

Table 9. Cases with two simultaneous significant antibody titer rises and corresponding results from antigen detection by TR-FIA.

	Number of cases		One finding by TR-FIA							Two findings by TR-FIA		
Significant antibody titer rises against	with serologic double infections	where TR-FIA ND	ADE	INA	INB	PIV	RSV	neg	sum	ADE & INA	INA & RSV	INB & PIV
ADE & INA	75	14	6	33	-	-	-	18	57	4		
ADE & INB	54	18	6	.	8	-	-	22	36			
ADE & PIV	38	13	14	.	1	-	-	10	25			
ADE & RSV	16	4	4	1	-	-	-	7	12			
ADE & MPn	32	12	5	1	-	-	-	14	20			
INA & INB	25	6	-	6	9	-	-	4	19			
INA & PIV	17	2	-	8	-	-	-	7	15			
INA & RSV	20	6	-	7	-	-	-	5	12		2	
INA & MPn	18	5	1	9	-	-	-	3	13			
INB & PIV	7	1	-	-	-	-	-	5	5			1
INB & RSV	0	0	-	-	-	-	-	0	0			
INB & MPn	2	1	-	-	-	-	-	1	1			
PIV & RSV	6	3	-	-	-	-	-	3	3			
PIV & MPn	19	7	-	-	-	1	-	11	12			
RSV & MPn	5	3	-	-	-	-	-	2	2			
Total	334	95	36	65	18	1	0	112	232		7	

Table 10. Cases with three simultaneous significant antibody titer rises and corresponding results from antigen detection by TR-FIA.

	Result by TR-FIA							
Serological triple infection detected	ADE	INA	INB	PIV	RSV	negative	ND	Total
ADE& INA &INB	1						3	4
ADE& INA &PIV		1						1
ADE& INA &RSV						2		2
ADE& INA &MPn		1						1
ADE& INB &PIV						1	1	2
ADE& INB &RSV							1	1
ADE& INB &MPn							1	1
ADE& PIV &RSV						1	1	2
ADE& PIV &MPn	1					1	1	3
INA & INB &MPn			1					1
INA & PIV &RSV		1						1
INB & PIV &Mpn						1		1
Total	2	3	1			6	8	20

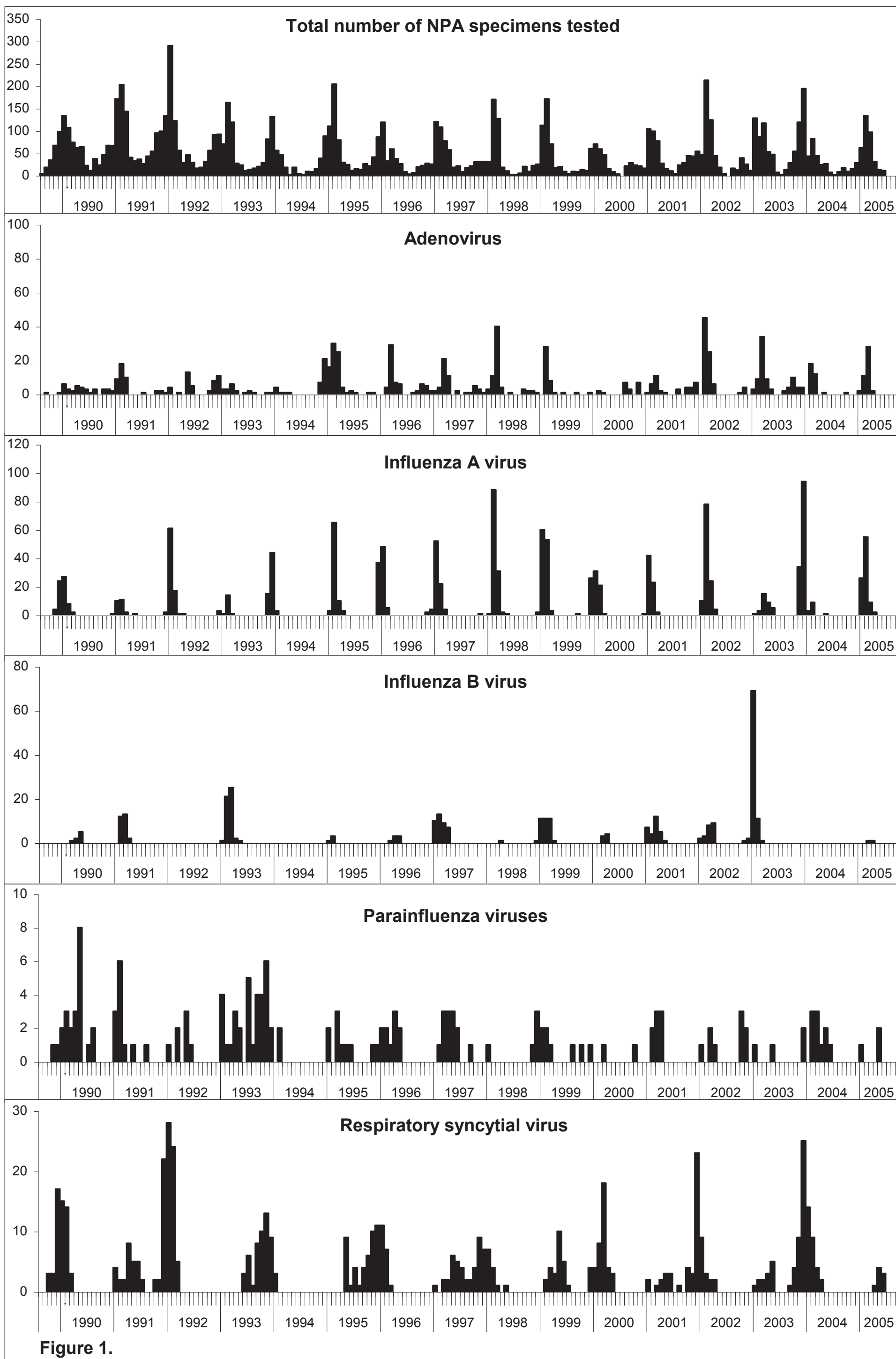

Figure 1.

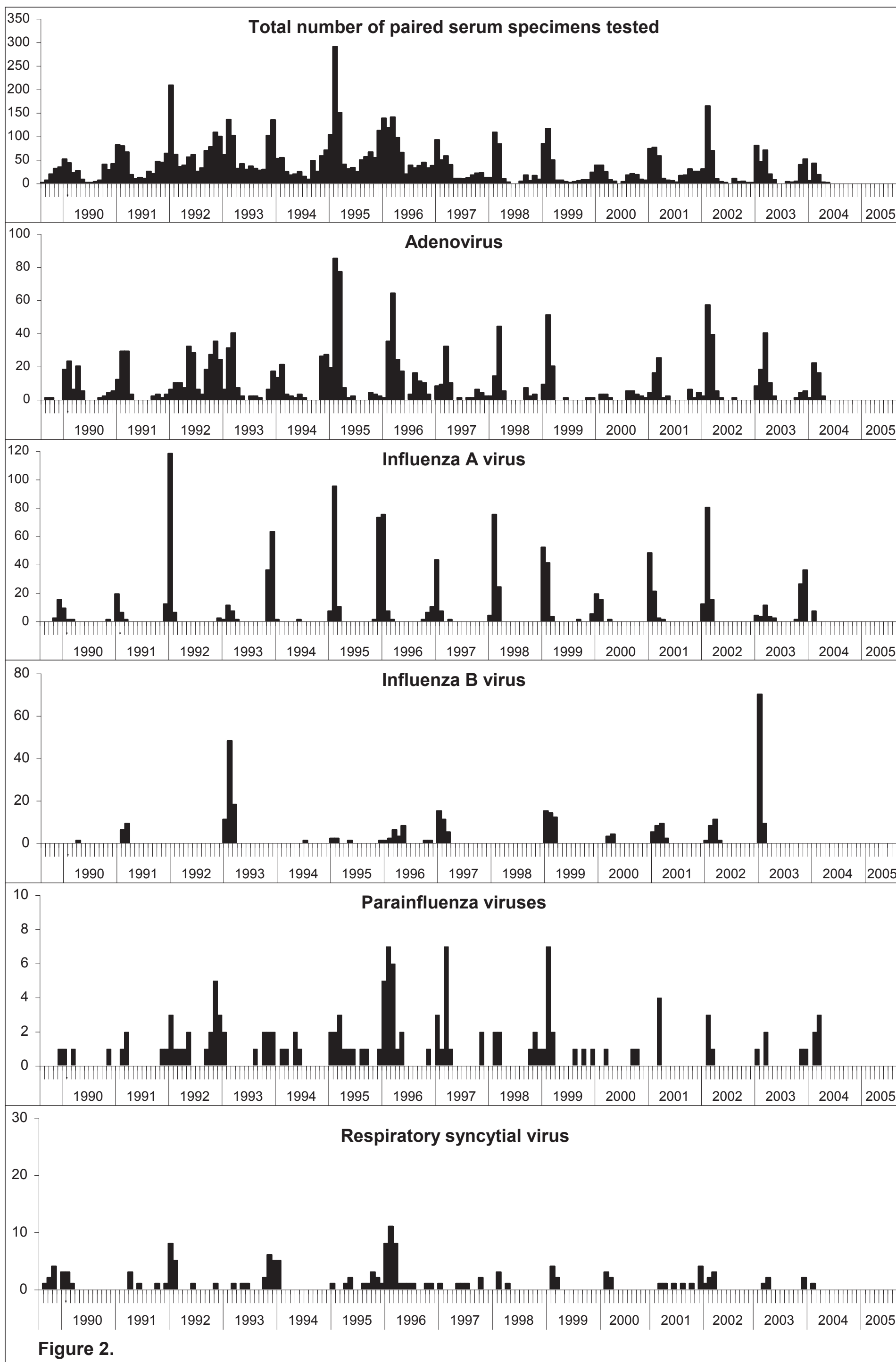

Figure 2.

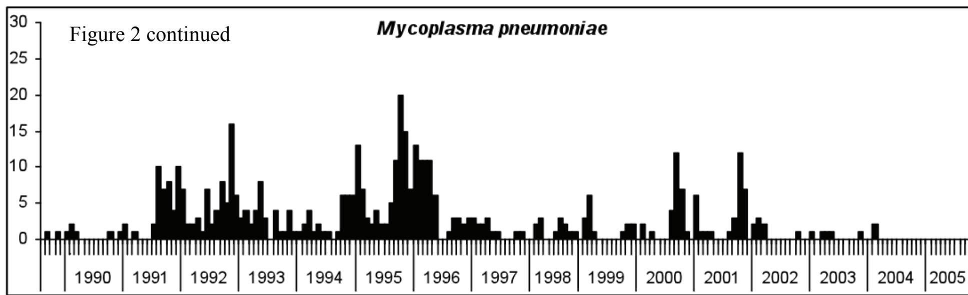

Figure 1. (*on page 52*) The seasonal occurrence of respiratory viruses in Finland over a period of 16 years from 1989 to 2005. Nasopharyngeal aspirates were collected from patients with an acute respiratory infection. The specimens were analyzed by TR-FIA for the presence of adeno-, influenza A-, influenza B-, parainfluenza-, and respiratory syncytial viruses. The bars in the top panel represent the total number of specimens analyzed each month and the bars in the pathogen-specific panels represent the number of positive findings each month.

Figure 2. (*on pages 53 and 54*) The seasonal occurrence of respiratory viruses and *M. pneumoniae* in Finland over a period of 16 years. Acute and convalescent phase sera were collected from patients with an acute respiratory infection. Antibodies to adeno-, influenza A-, influenza B-, parainfluenza-, and respiratory syncytial viruses and to M. pneumoniae were measured by the complement fixation test. The bars in the top panel represent the total number of serum pairs analyzed each month and the bars in the pathogen-specific panels represent the number of positive findings each month.

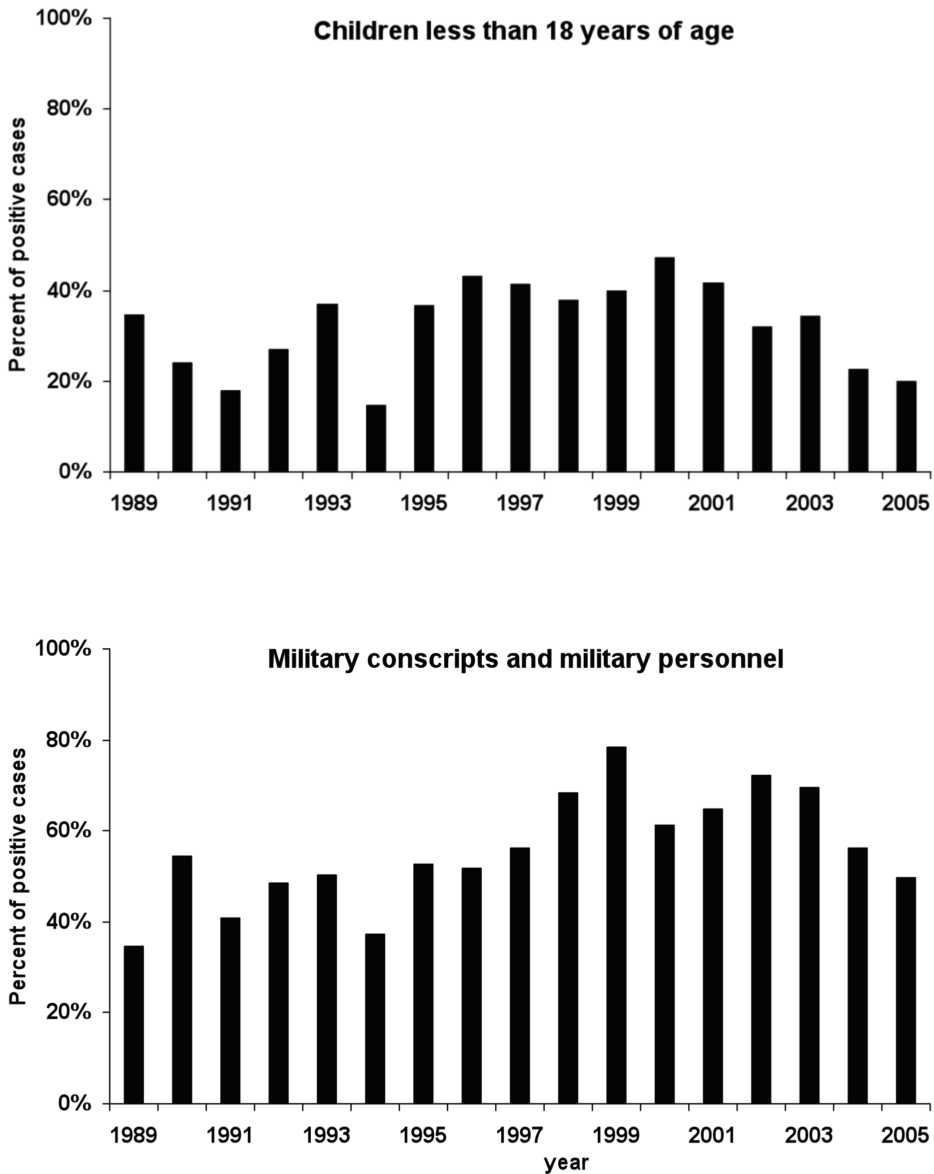

Figure 3. Percentage of positive laboratory findings from patients with acute respiratory infection. The results are presented separately for the two segments of the population from whom the highest number of specimens have been received over the 16 year observation period from 1989 to 2005, i.e., children less than 18 years of age and army conscripts and military personnel. Results from direct antigen detection by TR-FIA and serology by complement fixation are combined.

6 Discussion

In this thesis, molecular methods were developed for the detection of adenoviruses and *M. pneumoniae* from clinical specimens. These methods were thoroughly validated against conventional diagnostic methods using appropriately collected clinical samples from individuals with upper and lower respiratory tract infections. In addition, the TR-FIA method, developed at the Department of Virology, University of Turku, for the detection of seven common respiratory viruses, was introduced at the Laboratory of Respiratory Viruses of the National Public Health Institute in Helsinki. This method was applied for the rapid diagnosis of respiratory tract infections in different population groups. The diagnostic sensitivity and value of TR-FIA was evaluated in relation to CFT serology on several thousand patients. TR-FIA was used as primary method for the identification of respiratory viruses in this laboratory for over 16 years. Data obtained from this long-term study allow for an analysis of the temporal circulation of these pathogens in the Finnish population.

During the 1950s and 1960s many important respiratory viruses were identified. As better understanding of their clinical relevance and epidemiological behavior accumulated, the need for more rapid and sensitive diagnostic methods became obvious. Rapid diagnosis of viral respiratory tract infections was first attempted in the late 1950s in order to quickly identify influenza infections. The methods used at that time were virus culture and serology. Over the following decade the method of antigen detection by immunofluorescence staining was introduced, and is, after refinement, still in use in many laboratories. Specific reagents became available for the seven respiratory viruses that were believed to most frequently cause infections that require hospital treatment particularly of children: adenovirus, influenza virus types A and B, parainfluenza virus types 1, 2, and 3, and RSV. The classical studies of respiratory tract infections clearly demonstrated the importance of rhinoviruses and coronaviruses as causative agents of upper respiratory tract infections. However, these viruses were less frequently detected in hospitalized patients who apparently suffer from more severe infections. Only after sensitive and specific molecular methods became available in the late 1980s, a better understanding of the clinical significance of rhinoviruses and coronaviruses was obtained, and it is now clear that also these viruses can cause severe respiratory tract infections. Over the past 12 years, several previously unknown respiratory viruses have been detected and characterized. These include e.g. the human metapneumovirus, several coronaviruses (SARS, NL-63, HKU-1, MERS) and bocaviruses. Sophisticated molecular methods have played a central role in identification of these “novel” respiratory viruses, and these nucleic-acid based methods have quickly evolved into a corner stone in the virus diagnostic laboratory. Along with molecular methods, also immunological

methods for the detection of viral antigens evolved from fluorescent antibody testing via radio- and enzyme-immunoassay to TR-FIA and its more recent modifications. The TR-FIA method widely used in this thesis, proved to be a sensitive and specific technique that allowed for a rapid detection of these seven respiratory viruses at a reasonable cost.

6.1 Detection of adenovirus DNA in adult patients with respiratory infections by PCR (I)

Adenoviruses frequently cause outbreaks of respiratory infections particularly in army training centers. Sometimes these infections are clinically severe and require hospitalization of the patient. At the time when the samples for this study were collected, the Central Military Hospital was right next door to the laboratory, and close collaboration between the two institutions was established. Therefore, most samples have been collected at the Central Military Hospital, which also guaranteed optimal transport logistics for the specimens. At the time this study was performed, PCR was not yet established as a routine method for the detection of adenoviral infections. This study was one of the first to thoroughly compare several diagnostic methods, which helped to assess the true sensitivity of the adenovirus PCR described in study I.

Conventional culture in A549 cells with subsequent confirmation by indirect immunofluorescent staining and TR-FIA were performed on the fresh samples immediately after their arrival at the laboratory. A high proportion of the samples, almost 60 %, were found adenovirus-positive by these methods. Similar sensitivity was found by PCR. Interestingly, samples collected on the first day of illness, were more often positive by PCR than by conventional virus culture. This finding might be explained by the fact that early during the course of the illness mucus production might be less prominent as compared to a later phase. A similar difference was also observed on the 7th day of the illness or later. Disappearance of infectious virus during this stage of the disease likely explains this phenomenon. Rapid culture, antigen detection by EIA, and PCR were done on previously frozen samples. Although adenoviruses are non-enveloped viruses, even one cycle of freezing and thawing may affect the sensitivity of tests performed later on. As shown in this study, rapid culture, TR-FIA and EIA were considerably less sensitive than conventional culture and PCR. Inactivation or aggregation of the viruses during storage may, at least in part, explain this reduced sensitivity.

Obviously this freezing and thawing did not affect the yield of virus DNA for PCR testing because the extraction procedure is presumably efficient also for stored specimens. However, for rapid culture, TR-FIA, and EIA, the specimens were further diluted in order to have sufficient material available for all tests. With this dilution factor, samples containing only minor amounts of adenovirus may thus have remained undetected.

EIA detected slightly more positive samples than TR-FIA. The difference in these two test principles is that EIA is based on polyclonal guinea pig and rabbit antisera while the immunoreagent in TR-FIA was a mouse monoclonal antibody. The high specificity of mouse monoclonal antibodies may contribute to a decreased sensitivity if only minor changes in the recognized epitope accumulate.

Paired, acute and convalescent phase sera were available from less than half of the patients for testing by CFT. A minor fraction of patients with a positive adenovirus finding failed to mount a diagnostic antibody response. On the other hand, CFT serology identified several additional adenovirus infections that would have gone undetected by all other methods.

6.2 PCR for the detection of *M. pneumoniae* DNA in children with acute otitis media (II).

This is one of the first studies where *M. pneumoniae* was detected from MEF specimens of small children with acute otitis media. Culture of *M. pneumoniae* is cumbersome, labor-intensive, and often time-consuming, and therefore is of little value in the microbial work-up of patients with respiratory infections. Although the discussion continues whether or not to treat this frequent illness in small children with antibiotics, recent findings indicate that children receiving amoxicillin-clavulanate experience less often treatment failure as compared to children receiving placebo (Ruohola, Waris et al. 2009). The optimal choice of antibiotic may, however, require specific identification of the causative pathogen early during the course of the illness. To this end, PCR provides a sensitive, specific and rapid diagnostic method to detect *M. pneumoniae*.

The 380 MEF specimens for this study were collected between 1994 and 1997. Epidemiological records from the Finnish National Infectious Diseases Register (available since 1995) indicate that during the years 1995, 1996, and 1997 *M. pneumoniae* was identified throughout the year. However, the number of Mycoplasma cases diagnosed during these years was relatively small. This may be due to insensitive detection methods used in diagnostic laboratories at that time.

The P1 gene was selected as target for the PCR test. P1 is a highly immunogenic protein to which most infected individuals mount an immune response (Jacobs, Stuhlert et al. 1988; Fink, Read et al. 1995; Tuuminen, Suni et al. 2001). With 569 base pairs, the amplification product is unusually large. The primers were selected in order to clone and express parts of the P1 protein. For diagnostic purposes only, a shorter amplification product would have been chosen. Yet the specificity of the amplified product was verified by Southern hybridization using a 5’-[^{32}P]-labeled probe. Also the probe with 514 base pairs was, according to present criteria, uncommonly long. These long amplification products and probe may have affected the sensitivity of the *M. pneumoniae* DNA-specific PCR.

Of the 380 MEF specimens, 16 were found positive by the *M. pneumoniae* PCR. For several reasons the significance of these findings remains uncertain. Firstly, for only 7 of the 16 patients with a *M. pneumoniae* –positive PCR finding in MEF, an NPA was available. Of those 7 samples, only one contained *M. pneumoniae* as shown by PCR. Second, by CFT serology a ≥4-fold titer increase was observed in only two of the 16 patients. A few titer decreases were found by IgG and IgM EIA and one by CFT. Third, and probably most importantantly, 11 of the *M. pneumoniae* PCR-positive MEF contained one or more bacteria frequently found in MEF of otitis media patients. In addition, 3 of the 16 specimens also harbored a common respiratory virus. Without the detection of cultivable *M. pneumoniae* organisms it remains difficult to estimate its role in the pathogenesis of acute otitis media. *M. pneumoniae* could be passively transported to the middle ear through the Eustachian tube after the epithelium has been damaged by a preceeding virus infection and eventually by negative pressure in the middle ear as has been demonstrated in connection with rhinovirus infection (McBride, Doyle et al. 1989). *M. pneumoniae* has been cultured from two patients presenting with otitis media with effusion (Okazaki, Akema et al. 1989).

6.3 The sensitivity of *M. pneumoniae* PCR is greatly influenced by the specimen type (III).

During winter 2001/2002 *M. pneumoniae* caused outbreaks in garrisons. At that time, most patients with lower respiratory tract infections requiring hospital treatment were referred to the Central Military Hospital in Helsinki. This provided an excellent opportunity to compare different types of clinical specimens for the diagnosis of *M. pneumoniae* by PCR. A diagnosis of *M. pneumoniae* infection could be established for 22 of the 32 patients. In this relatively small group of patients, sputum appeared to be the optimal sample type, as *M. pneumoniae* was detected in all 22 sputum samples. NPAs and throat swabs yielded only 16 and 12 PCR positive findings, respectively. On X-ray examination, pneumonia was identified in all 32 patients. One can therefore assume that the infection process was still ongoing in the lungs at the time these specimens were collected. From these young military recruits, it was relatively easy to obtain a sputum sample that likely contained mucus coughed up from the lower respiratory tract with little saliva contamination.

From children and from the frail elderly it is obviously more difficult to obtain a suitable sputum specimen. There is ongoing controversy about the optimal respiratory specimen for the detection of *M. pneumoniae.* Nasopharyngeal aspirates, nasopharyngeal and oropharyngeal swabs have been used as alternative samples. In a group of 42 children with an average age of 5 to 6 years and an X-ray confirmed lower respiratory infection, 21 were diagnosed with an *M. pneumoniae* infection based on serological findings by EIA. Of these 21 children, 12 were found positive

by PCR, 9 from the oropharyngeal swab and 9 from the nasopharyngeal swab (Michelow, Olsen et al. 2004). In a comparison of NPA and throat swabs, the latter sample proved to be slightly superior, mainly due to the fact that one third of the specimens contained inhibitors of PCR (Reznikov, Blackmore et al. 1995). Similar findings have been presented in another study, but there the difference was not statistically significant, due to the small number of patients (Gnarpe, Lundback et al. 1997).

In the present study, the serological results strongly support the PCR findings. Acute and convalescent serum pairs were available from 20 of 22 patients with a PCR-positive result from the sputum specimen. Of those 20 patients, 15 had a ≥ 4-fold titer increase and the remaining 5 patients had a CF titer of ≥256 already in the acute phase serum. Studying a large group of children with X-ray-confirmed pneumonia, IgM and IgG serology performed on acute and convalescent phase sera, proved to be much more sensitive than PCR performed on NPA collected during the acute phase of illness (Waris, Toikka et al. 1998). However, it appears based on the present and other studies that a combination of several clinical samples and laboratory methods is required in order to achieve optimal accuracy of *M. pneumoniae* diagnosis.

6.4 Inclusion of serological methods significantly increases diagnostic findings in epidemiological studies of acute otitis media in children (IV)

Acute otitis media is a frequent complication of viral respiratory tract infections in children. The FinOM Studies in the 1990s provided an opportunity to collect several types of clinical samples including MEF, NPA, and serial serum samples for a thorough diagnostic work-up of the otitis events in children. Presently, with the availability of molecular methods and of methods for rapid antigen detection, serology is rarely used for the identification of pathogens causing upper respiratory tract infections and their complications.

In a carefully conducted study published 13 years ago (Heikkinen, Thint et al. 1999), an almost similar number of AOM as in the present study (IV), were virologically, bacteriologically, and serologically analyzed (Table 11).

The age of the children included in the study by Heikkinen and colleagues (Heikkinen, Thint et al. 1999) was from 2 months to 7 years, while in the present study (IV), the children were ≤2 years of age. Heikkinen and co-workers used virus culture, virus antigen detection by immunofluorescence staining, and serology on acute and convalescent phase sera likewise done by fluorescent antibody

Table 11. Comparison of two rather similar acute otitis media studies in children.

	Räty et al. 2004	Heikkinen et al. 1999
No. of patients	447	456
Research period	1994 - 1997	1989 - 1993
Age range	2 months to 2 years	2 months to 7 years
Virus in MEF	43	77
Viral infection[1]	163	168
Adenovirus	32	23
Influenza viruses	17	24
Parainfluenza viruses	42	29
RSV	72	65
Enteroviruses	N.D.[2]	27

[1]Combination of results obtained from TR-FIA antigen detection and CF serology (Raty, Ziegler et al. 2004) including adenovirus, influenza A and B, parainfluenza viruses types 1, 2, and 3, and RSV; fluorescent antibody staining, virus culture, and serology (Heikkinen, Thint et al. 1999) including adenovirus, influenza viruses, parainfluenza viruses, enteroviruses, and RSV, respectively. [2]N.D. = not done

staining. Both studies have been conducted over several years and the distribution of viruses identified in these AOM events was astonishingly similar, although the study sites were geographically distinct. The findings of the studies are summarized in Table 11.

The importance of *M. pneumoniae* as a possible cause of AOM has been discussed above (II). The present study focused on common respiratory viruses for which suitable laboratory tests were available. As shown by Heikkinen and colleagues (Heikkinen, Thint et al. 1999) and many others, a variety of other viruses can be detected in MEF samples when appropriate methods are applied (Nokso-Koivisto, Raty et al. 2004; Rezes, Soderlund-Venermo et al. 2009; Marom, Nokso-Koivisto et al. 2012; Ruohola, Pettigrew et al. 2013; Stockmann, Ampofo et al. 2013).

6.5 Long-term laboratory surveillance of several important respiratory pathogens (this thesis)

Over a period of 16 years, respiratory infections in certain segments of the Finnish population were monitored through virus- and *M. pneumoniae*-specific laboratory analyses. A total of 12,020 infection episodes of ARI or ILI were included. In 5,343 patients (44.5 %) a respiratory virus or *M. pneumoniae* could be identified as a possible etiological agent either by antigen detection and/or by serology. A primary purpose of this surveillance was to collect specimens needed by the NIC in order to determine antigenic and genetic characteristics of circulating influenza viruses.

Children and young adults were overrepresented in this surveillance. This surveillance system was originally established in order to fulfill the responsibilities of the NIC of THL, i.e., to collect suitable clinical specimens for isolation and characterization of seasonal influenza viruses. Since respiratory viruses spread efficiently in daycare centers and in garrisons, a large fraction of the specimens analyzed were from pediatric clinics and from military health care services. Some samples from adults were sent from occupational health care centers. The remaining specimens were contributed by public and private health care centers serving patients of all age groups.

Influenza A epidemics of various size occurred during each year of observation. Often the first influenza cases were identified in October or November, but major epidemic activity was usually observed from January to March. Two exceptions to this were the years 1993 and 2003 when the epidemic peak occurred already in December or November/December, respectively. In these years new antigenic variants A/Beijing/32/92 (H3N2) and A/Fujian/411/2002 (H3N2), respectively, had started to spread internationally during the previous spring. In contrast to influenza A, there were five winter seasons with very few or no influenza B infections.

In the spring of the years 1998 and 2005 single cases of influenza B findings were observed in the absence of a preceding epidemic in the country. It remains unclear whether travellers arriving from countries with influenza B activity have imported these cases. During five seasons, influenza A and influenza B were circulating simultaneously (Fig. 1).

Most of the adenovirus infections occurred in garrisons. Adenovirus infections were recorded almost every month of the year. Major adenovirus activity appeared before or simultaneously with influenza epidemics.

While in the United States RSV epidemics occur annually (Langley and Anderson 2011), in Finland major RSV epidemics are observed at two year intervals during the winter period, with a minor outbreak in the preceding spring (Waris 1991). In the present study, this biannual cycle was less pronounced and could be identified in the seasons 1991/1992, 1999/2000, 2001/2002, and 2003/2004. While most of the RSV-positive samples were from children, some cases were noted among military recruits and in other adults. Parainfluenza infections occurred both in children and in adults. The epidemic timing of these viruses was less pronounced than that of RSV. A survey in the United States revealed that parainfluenza virus type 1 caused epidemics every two years in the late summer and fall, type 2 caused only sporadic outbreaks and type 3 appeared every year with peak epidemic activity often during the summer months (Hall 2001). In the present analysis parainfluenza virus type 3 was the most frequently detected type based on antigen detection. Most of the observed parainfluenza cases were sporadic in children with neither major epidemics nor with years of high epidemic activity.

Infections with *M. pneumoniae* were diagnosed in all age groups. However, since paired sera from small children were less frequently available, young adults and

individuals > 30 years, were overrepresented in this analysis. In the present study, direct detection methods for *M. pneumoniae* were not available, nor was IgM serology used, which appears to be the most useful diagnostic test to identify *M. pneumoniae* cases particularly in children (Waris, Toikka et al. 1998). However, serological analysis clearly showed significant yearly and seasonal variation in *M. pneumoniae* infections.

In order to rapidly identify an etiologic agent in clinical specimens, methods for the direct detection of a pathogen are essential. In certain clinical cases and for epidemiological surveillance the value of serological methods should, however, not be underestimated. Restricting data analysis in the present study to viruses only, serology yielded significantly more positive findings than antigen detection. Most impressively, adenovirus CF test yielded more than twice as many significant titer increases compared to the findings by TR-FIA in young adults. Adult patients likely seek medical assistance later during the course of an infection, at the time when the pathogen may already have been cleared from the upper airways but a diagnostic rise in the antibody levels can still be detected between acute and convalescent serum specimens. In addition, the antigen detection tests for adenoviruses appear to be suboptimal (Kunz and Ottolini 2010). For patients from whom NPA and paired serum specimens were available, serology produced clearly more diagnostic findings than TR-FIA with the exception of RSV. RSV infections typically occur in young infants, in whom the seroresponse to the infecting pathogen may be slow. With the sampling strategy used in this surveillance, diagnostic titer rises may therefore have gone undetected. It has been shown previously (Falsey and Walsh 2000) that serology is not a particularly sensitive and reliable method for identification of RSV infections.

Combining antigen detection with serology surprisingly often revealed more than one etiological agent during one episode of respiratory infection. Multiple infections were particularly frequent among military recruits, with adenovirus and influenza being mostly involved. In 35 of the more than 9,000 NPAs investigated, two viruses were identified. Living conditions in garrisons and day-care centers may create significant risk for respiratory infection, and pathogens can thus spread efficiently, causing concurrent outbreaks or subsequent infection episodes (Owens, Canas et al. 2009). The number of daycare children included in this study may be too small to make estimations whether a similar frequency of infections with multiple viruses could be established. Using sensitive molecular methods for the diagnosis of upper respiratory infections, multiple microbial findings are common (Ruohola, Waris et al. 2009), often making it difficult to determine the pathogen responsible for the symptoms or how each of the pathogens contributes to the clinical presentation. Surprisingly, a recent study has shown that simultaneous virus findings correlate with less severe disease (Martin, Kuypers et al. 2011). On the other hand, young children with viral coinfections seem to experience more severe forms of pneumonia as compared to children with a single viral pathogen identified (Cilla, Onate et al. 2008).

In different age groups of the present study material the spectrum of viral pathogens varied. While in the age group ≤17 years, RSV, influenza A, adenovirus, and parainfluenza viruses predominated, adeno- and influenzaviruses were most often found in young adults. Adenoviruses have long been identified as common respiratory pathogens in military training centers often causing severe infections (Gray, Goswami et al. 2000; Gray 2006; Burke, Vest et al. 2011). In individuals over 30 years of age, influenza A and B viruses were the pathogens most often detected, emphasizing the public health importance of influenza viruses in this section of the population. Also here, the uneven representation of age groups in this surveillance may have contributed to this finding.

During the years this surveillance was conducted, antigen detection assays have been the cutting-edge technology for the rapid identification of respiratory pathogens in clinical samples. The sensitivity of the TR-FIA method compared well with other standard laboratory techniques (Halonen, Herholzer et al. 1996). Molecular methods have found their place in diagnostic laboratories in the late 1990s or later, and only with the availability of multiplex methods has this technology been more widely used for diagnostic and surveillance purposes. Both TR-FIA antigen detection and CF serology have been limited to viruses for which suitable diagnostic reagents have been available. Thus other important respiratory viruses, including rhino- and enteroviruses, parechoviruses, coronaviruses, human metapneumoviruses, and bocaviruses, have not been included in the present study. However, the results presented here show that these viruses and *M. pneumoniae* cause a significant fraction of acute respiratory tract infections in different segments of the Finnish population. Many national and international surveillance systems are limited to influenza viruses. Since none of the pathogens investigated in this study can reliably be identified by specific clinical symptoms, more broad-spectrum diagnosis and surveillance of respiratory pathogens is essential in order to make more accurate clinical decisions and use more efficient preventive strategies.

7 Conclusions

The studies presented in this thesis were initiated soon after sensitive and specific, practical and user-friendly methods for the rapid identification of respiratory viruses in clinical specimens became available. The TR-FIA, a central method in all studies presented, has been developed at the Department of Virology, University of Turku, in the early 1980s. This method has been transferred to and thoroughly validated at the Virology Unit of THL. Using this TR-FIA technique it has been possible to study the seasonal occurrence of certain respiratory viruses in different segments of the Finnish population, and to establish a laboratory-based surveillance of these viruses. This activity, among others, provided the NIC within the Virology Unit with suitable specimens for the isolation, identification, and characterization of influenza viruses circulating in Finland. As expected, RSV was the most commonly detected virus in children. But also other respiratory viruses were frequently found in this age group. Adenoviruses and influenza viruses were predominant in military recruits. The long-term surveillance study presented here does not reflect the composition of the entire Finnish population. A number of important respiratory viruses such as rhinoviruses, coronaviruses, hMPV and HBoV were not included in this surveillance. Thus, this surveillance does not provide a comprehensive epidemiological picture of the respiratory viruses in Finland. Yet, particularly child day-care centers and army garrisons offer optimal conditions for an efficient spread of respiratory pathogens. Nevertheless, a more systematic sampling strategy covering all age groups and geographic areas is currently being established.

The TR-FIA method has been available for a limited number of respiratory viruses. This included the seven viruses previously thought responsible for a significant fraction of more severe infections particularly in children. Several previously unknown respiratory viruses, such as the human metapneumovirus, bocaviruses, and “novel” coronaviruses, have been identified in the past 12 years. In addition, during recent years, better understanding of picornaviruses as causative agents of upper and lower respiratory tract infections has accumulated. With the availability of highly sensitive and specific molecular methods, the development of immunological techniques for the detection of these viruses has been slow. TR-FIA is still a practical method that allows for testing of a considerable number of specimens. Results can be obtained in less than two hours after receipt of the specimen. Although molecular methods are increasingly becoming available in diagnostic laboratories, methods for rapid antigen detection will maintain an important role in the rapid diagnosis of respiratory infections. After completing the 16-year surveillance study presented here, TR-FIA has been replaced by multiplex PCR methods for the detection of respiratory infections in the Virology Unit.

The inclusion of serological methods for the diagnosis of respiratory infections has significantly increased the number of relevant findings in the 16-year surveillance presented here. Although it rarely produces a conclusive result in the acute phase of the disease, the value of serology should not be underestimated when performing epidemiological studies. Particularly from small children, it may be difficult and impractical to obtain venous blood samples for the diagnostic purposes in uncomplicated respiratory infections. However, novel laboratory methods for the identification of specific antibodies are becoming available, which possibly could be performed by capillary blood or alternative specimens. This may provide new opportunities in the future for the use of serology. CF technology has been used in the 16-year surveillance. Although immunoassays may be more sensitive, the CF method continues to be a valuable, high throughput screening method.

Combining methods for virus antigen detection with serology revealed a considerable number of respiratory infections with the involvement of more than one pathogen. Such infections have been particularly observed in young children and in military conscripts. This again underlines the fact that the spread of infectious pathogens is enhanced in such environments.

All the laboratory tests used in this study have their limitations. Every laboratory test produces occasionally results in the grey-zone, which makes interpretation difficult or even impossible. Since this work was primarily perfomed for surveillance purpose rather than as clinical diagnostic service, a new specimen was requested only in exceptional cases. Without any doubts, false-negative results have been reported. Borderline-positive results obtained by TR-FIA were further evaluated by a confirmatory test. Unclear serological results were verified by repeating the test. PCR results were based on reading the agarose gel in UV light or hybridization blots. In some cases subjective interpretation in such readings is unavoidable. In laboratory medicine, laboratories produce test results. However, the final diagnosis is established by the treating physician who has the overview of all clinical findings and laboratory test results.

Laboratory surveillance of microbial pathogens does not only help to identify the causative agent and direct doctors towards optimal treatment, but it may also help to identify the beginning of an epidemic with known or even with previously unknown pathogens.

8 Acknowledgements

The present study was carried out in the virus laboratories of the National Institute for Health and Welfare, former National Institute of Public Health.
I wish to express my warm thanks to

Professor Tapani Hovi for creating a research-friendly environment, his support and for giving me the opportunity to participate in the FinOM studies;

Professor Ilkka Julkunen for his never-failing enthusiasm and optimism even during hard times and turmoil and giving valuable advice also as one of my supervisors;

Docent Marjaana Kleemola and Docent Thedi Ziegler, for sharing their profound knowledge with me and for expert supervision of this work, their help and advice;

Docent Carita Savolainen-Kopra for arrangements and support during the final preparation of this work;

The official reviewers of my thesis, Docent Maria Söderlund-Venermo and Docent Hanna Tuokko for their constructive critisicism and most valuable comments;

Professor Kari Keinänen, University of Helsinki, for his kind guidance during the final stage of this study;

Anja Waroma, Sirkka Nyman, Annamari Harberg and Esa Rönkkö for exemplary, enthusiastic, reliable and never-failing assistance and continued friendship;

Marja Leena Kantanen for introducing me to virusserology and Liisa Piirainen for help and support and preparing the antigen detection test for use in the laboratory;

Saara Nygren and Sirpa Passoja for teaching me in the field of data processing and analyses;

Jukka Lindeman and his colleagues in the library for expert and never-failing help with all requests;

Pia Korkeamäki, Sanna Koivumäki, Seija Puro for valuable technical help in the final preparation of the thesis;

All my colleagues and friends at the Institute for help and support;

My friends outside the Institute for patience when I was seldom available but was busy writing the various versions of this thesis;

Miika, Pauliina and Miikka for optimism and encouragement, you are most important and dear to me.

Herttoniemi, September 2013

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