Respiratory Syncytial Virus

Improving surveillance and diagnostics in Europe

Tamara Meerhoff

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Respiratory Syncytial Virus

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There are two devices which can help the sculptor to judge his work: one is not to see it for a while. The other... is to look at his work through spectacles which will change its color and magnify or diminish it, so as to disguise it somehow to his eye, and make it look as though it were the work of another...

Gian Lorenzo Bernini -lived 1590-1680

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1

General introduction

General Introduction

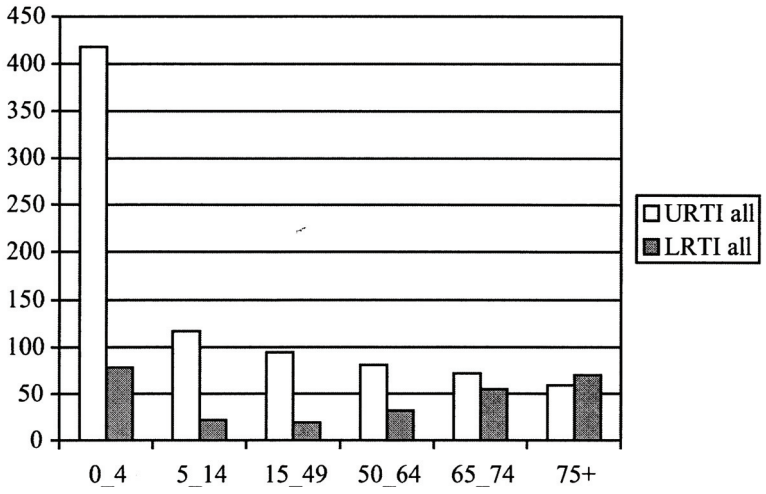
The studies presented in this thesis address how surveillance of respiratory syncytial virus (RSV) can be improved, investigate determinants of seasonal RSV activity, and assess diagnostic methods. In this introductory chapter, the background on respiratory illnesses and RSV in particular are described, as well as diagnostic and surveillance methods. Finally, the aim, relevance and outline of the thesis are presented.

Acute Respiratory Illness

Acute respiratory tract infections are the most common illness in all individuals: young children have about 4-8 respiratory tract episodes per year, for older children the incidence is slightly lower with 2-6 episodes per year.[1] In persons older than twelve years of age respiratory illness also occur frequently, 37% of persons reported to have had respiratory tract symptoms in the last two months.[2]

Acute respiratory tract infections frequently lead to consultation in general practice (GP). A substantial proportion of complaints (13%) in general practice concerns the respiratory tract and acute infection of the upper airways is with a prevalence of 57/1000 patients per year the second most common complaint presented to the general practitioner.[2] The majority of respiratory infections affect the upper respiratory tract, and are mild, self-limiting viral upper respiratory tract infections (URTI).[3] However, for lower respiratory tract infections (LRTI), each year about 3% of all children less than 1 year of age need to be admitted to hospital.[4] The incidence of complaints concerning the respiratory tract, specified for URTI and LRTI by age group is presented in Figure 1.1.[5]

Figure 1.1: Incidence rates (per 1000) of GP-diagnosed upper respiratory tract infections (URTI) and lower respiratory tract infections (LRTI) according to age in years



Source: Hak et al., Family Practice, 2006

Respiratory disease causes a substantial burden on patients and their families. Also in terms of economical burden on society the costs are high due to consultations with doctors, direct medical costs, and the indirect costs of missed days from work or absences from school and day care.[6,7] Costs attributable to respiratory tract infections in both outpatient and inpatient settings have an important impact on healthcare budgets. High risk groups to develop severe illness are the very young, immuno-compromised patients, patients with underlying chronic illness, and the elderly.[8-12]

Aetiology of respiratory tract infections

A great variety of viruses can cause respiratory tract disease, e.g. respiratory syncytial virus (RSV), influenza virus, parainfluenza virus, rhinovirus, coronavirus, and the human metapneumovirus. In addition, three new viruses have recently been discovered: human bocavirus, KI polyoma virus and WU polyomavirus.[13-15] The aetiology of acute respiratory tract infections has been investigated in infants in the Netherlands. Rhinovirus was most frequently detected in cases, followed by RSV and coronavirus.[16]

Common respiratory viruses, virus family and estimated rates of hospitalisation are presented in Table 1.1. Other pathogens such as enteroviruses, adenoviruses and the bacteria *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* can also cause respiratory symptoms and pneumonia. In this thesis we will mainly focus on RSV.

Table 1.1: Common respiratory viruses and estimated hospitalisation rates for children below the age of five

Pathogen	Virus family	Estimated rates of hospitalisation
Respiratory syncytial virus	Paramyxoviridae	0.3-5.2/1000 children [17-19]
Influenza virus	Orthomyxoviridae	0.6-1.4/1000 children [17;18]
Rhinovirus	Picornaviridae	4.8/1000 children [20]
Coronavirus	Coronaviridae	0.6-2.2/1000 children [21;22]
Parainfluenza virus	Paramyxoviridae	1.2/1000 children [18]
Human metapneumovirus	Paramyxoviridae	1.3/1000 children [17]

Clinical symptoms are somewhat similar for various respiratory pathogens and often include common cold and influenza-like symptoms (Table 1.2).[23] Therefore it is difficult to define the cause of illness when looking at clinical symptoms alone.[24] The aetiology of respiratory disease can be assessed by laboratory testing. Hereby the relative burden of disease caused by the different respiratory pathogens can be better defined. It is important to determine the cause of illness by laboratory diagnosis, because it provides insight into the aetiology, specific treatment can be applied and preventive measures can be implemented.

Table 1.2: Common respiratory viruses and their associated clinical syndromes

Respiratory virus	Clinical manifestations	Clinical complications
Respiratory syncytial virus	A feverish influenza-like illness with cough and wheeze	Acute breathing difficulties
Influenza virus	Illness usually with fever, cough, sore throat, muscle pains	Otitis media and pneumonia
Rhinovirus	Common cold	Occasionally causes pneumonia
Coronavirus	Usually a mild form of influenza-like illness	Sinusitis and pneumonia
Parainfluenza virus	Wide range of symptoms ranging from common cold to croup	Viral pneumonia and acute breathing difficulties
Adenovirus	Common cold symptoms with vomiting	Can cause sinusitis and occasionally lead to pneumonia

Source: Health Protection Agency. A Winter's Tale.

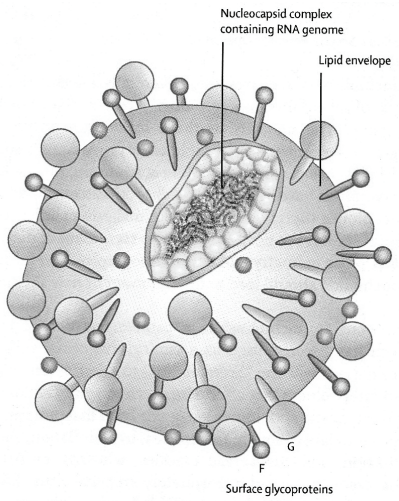
Respiratory syncytial virus

In 1956 a new virus was discovered in a chimpanzee with respiratory symptoms and it was named chimpanzee coryza agent.[25] In the following decade the virus was renamed respiratory syncytial virus (RSV) to reflect the giant syncytia that were formed in tissue culture and has been known to be the most important viral pathogen of respiratory tract infection in infants and young children.[26,27]

RSV is often introduced into the home by school-aged children who are infected with RSV and have a mild upper respiratory tract infection.[28] Viral transmission occurs by direct inoculation of contagious secretions from the hands by large-particle aerosols into the eyes and nose, but rarely the mouth.[29] The prolonged survival of RSV on skin, cloth, and other objects emphasizes the importance of fomites in nosocomial spread.[30] After an incubation period of 2-8 days, RSV replicates in the nasopharyngeal epithelium, with spread to the lower respiratory tract one to three days later. The characteristic inflammation of RSV bronchiolitis is necrosis and sloughing of the epithelium in the small airways, with oedema, and increased secretion of mucus, which obstructs flow in the small airways.[10]

RSV is a single-stranded RNA virus. The genomic RNA is packaged in a shell of proteins, the nucleocapsid, which is surrounded by an outer envelope of the virus that consists of viral glycoproteins embedded in a lipid bilayer (Figure 1.2).

Figure 1.2: Schematic diagram of RSV, illustrating surface glycoproteins, lipid envelope and the nucleocapsid complex [31]



Source: Smyth and Openshaw, Lancet 2006.

There are two major groups of RSV strains, A and B, which are distinguished mainly by the variations within the G protein. The G (attachment) protein is important for viral attachment and release. The F (fusion) protein is important for viral attachment. Other structural proteins are the L (large polymerase complex) protein, P (phosphoprotein), N (nucleoprotein), M (matrix), SH (small hydrophobic) protein, and the small envelope (M2).[10]

Epidemiology of RSV

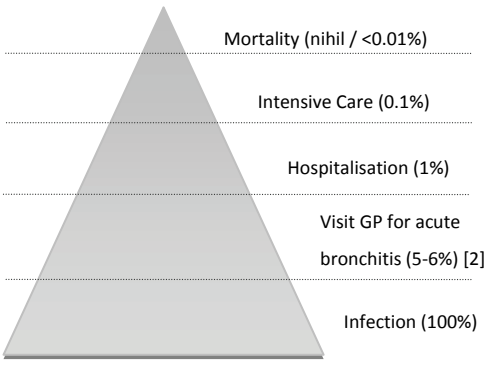
RSV is the most important viral pathogen in acute respiratory tract infections in children under 2 years of age. The infection rate of children aged less than 12 months is estimated at 69/100 children, virtually all children have been infected by the age of 24 months and about half had experienced two

infections.[32] The risk of re-infection is inversely related to the level of neutralizing antibodies in the serum and re-infection illnesses are generally mild.[32] The spectrum of clinical disease ranges from mild upper respiratory tract illness, otitis media, croup, to apnoea in premature infants, pneumonia and bronchiolitis.[33] In Europe, RSV accounts for 42-45% of hospital admissions for lower respiratory tract infections in children younger than two years of age, and inpatient populations tend to be younger and experience greater disease severity.[34] For RSV bronchiolitis lengths of stay in European hospitals range from 4 to 10 days. Premature babies born at 30-35 weeks of gestation, infants with congenital heart disease, HIV-infected subjects, and patients on intensive immunosuppressive therapy are considered to be at risk of increased mortality and morbidity during RSV infection.[12,35-37] It has been estimated that RSV causes about 500,000 deaths in children each year globally; while mortality due to RSV is very low in Western Europe, the number of child deaths due to acute respiratory infections worldwide is considerable with 70% of deaths occurring in Africa and Southeast Asia.[38-39]

Many of the studies that assessed the relation between respiratory illness and viral pathogens include hospitalised children as subjects. This provides limited information on the overall epidemiology, as hospitalised children constitute the peak of the iceberg.[40] The iceberg concept of infection is presented in Figure 1.3.

Recognition of re-infection with RSV is confounded by the fact that other respiratory viruses cause similar clinical symptoms. The frequency of RSV re-infections throughout life indicated that a large susceptible population is consistently available and that these usually mild re-infections are the primary source of serious infections in infants and those with underlying medical conditions.[10] The clinical impact in certain adult populations is considerable and RSV can be an important cause of winter mortality.[26] Those that are at increased risk for serious illness include adults with underlying cardiopulmonary disease, frail elderly persons, and the severe immuno-compromised.[9,42] RSV-related disease is often not recognized in elderly. This is probably due to the low awareness of RSV as cause of respiratory illness by the general practitioner and diagnostic testing is rarely performed.

Figure 1.3: Iceberg concept of RSV infection, epidemiological distribution of RSV infection in young children [41]



Source: Adapted from Bont, Caravisie 2004.

RSV epidemic patterns differ by geographic location. In countries with temperate climates, such as the Netherlands, RSV causes yearly epidemics during late fall, winter and spring.[43,44] In addition, a biannual pattern has been identified in some countries.[45,46] RSV can circulate all year round in equatorial countries and appears regularly in tropical or semitropical countries, but with different patterns of seasonality.[47] Epidemics frequently start in coastal areas or areas surrounded by water and then move to inland areas in the subsequent months.[47] The seasonality of RSV epidemics may be partly explained by weather-related behaviour. For example indoor crowding occurs in wintertime and may impact epidemics. Additionally, the immunity of the at-risk population is important as well as the transmission capability of the virus under certain weather circumstances.

An overview of the main features of RSV are presented below.[31]

Features of RSV infection:

- Cause of worldwide annual epidemics
- Infects almost all children by the age of two
- Responsible for about 70% of cases of bronchiolitis in children
- Causes coughs and cold in older children and adults
- Causes re-infection despite the presence of serum antibody
- The same serotype re-infects children and adults
- Associated with recurrent wheeze for many years after bronchiolitis

Source: Smyth and Openshaw. Lancet 2006

Prevention and treatment

At present, there is no licensed RSV vaccine. In the 1960s, a formalin-inactivated RSV vaccine was given to infants and children.[48] However, the vaccine did not protect against infection and was associated with an increased risk of severe RSV disease when some of the vaccinated children became naturally infected. A successful RSV vaccine should protect against bronchiolitis and pneumonia. However, it is unlikely that a RSV vaccine would protect against RSV infection because the naturally acquired immunity after RSV infection is neither complete nor persistent.[49] Nevertheless, protection against severe disease develops after primary infection. Different vaccines are under preparation for different populations at risk.[50,51]

Profylactic use of neutralizing antibody for high risk groups is available. Monthly administration of RSV hyperimmune globulin or monoclonal antibody against F protein (palivizumab) in premature infants or infants with chronic lung disease reduced the risk of subsequent hospitalisation for RSV infection.[52] Infants born with congenital heart disease are at risk for severe RSV infection, and palivizumab reduces RSV-related hospital admissions by about 45% in these children.[53] Palivizumab is the first effective humanized monoclonal to be used to prevent infection. However the cost is high and although palivizumab reduces hospital admissions for serious RSV disease, its cost-effectiveness for infants born at more than 32 weeks of gestation is not proven. In 2009, the recommendations for use of palivizumab have been updated by the American

Academy of Pediatrics to ensure optimal balance of benefit and cost from this expensive intervention.[54] Recently motavizumab, an enhanced-potency monoclonal antibody for the prevention of RSV has been evaluated. Pre-clinical data indicated that motavizumab has a much higher affinity for the F protein of RSV than palivizumab and is approximately 20-fold more potent than palivizumab in microneutralization studies.[55]

Currently the only therapy for RSV infection is aerosolized or oral ribavirin, a broad-spectrum antiviral agent that is approved only for hospitalised infants. Trials of ribavirin for RSV lacked sufficient power to provide reliable estimates of the effects. The cumulative results of three small trials show that ribavirin may reduce the duration of mechanical ventilation and may reduce days of hospitalization. In addition, use of ribavirin may be associated with a decrease in the long-term incidence of recurrent wheezing following RSV disease.[56] In practice, treatment of infants is generally limited to supportive care, including giving appropriate fluid replacement and oxygen.

Furthermore, antibiotics are regularly prescribed for acute respiratory infections such as bronchiolitis, although these are not recommended unless there is concern about complications such as a secondary bacterial infection. No evidence was found to support the use of antibiotics for bronchiolitis.[57,58]

Routine surveillance

Many countries in Europe have developed systems for the reporting of infectious diseases. The aim of such reporting systems has always been to discover epidemic outbreaks of infectious disease quickly, and the rationale is that while one doctor might only see one or two cases of an epidemic, collective reporting at a regional or national level will make it possible to see the full picture.[59]

Disease surveillance can be defined as the ongoing systematic collection and analysis of data and the timely dissemination of information to those who need to know so that action can be taken.[60] The general principle of surveillance is that each country has a list of notifiable diseases; this disease is required by law to be reported to government authorities.

One of the notifiable diseases that must be covered by the European Community Network for surveillance is influenza.[61] National networks for the

surveillance of influenza have existed in Europe since the 1950s. In the late 1980s, efforts were made to improve the clinical reports from sentinel physicians by integrating virological surveillance systems and by collecting data on a European level. The first European influenza surveillance project was the Eurosentinel scheme (1987-1991), this was followed by the ENS-CARE Influenza Early Warning Scheme (1992-1995, and the European Influenza Surveillance Scheme (1996-2008).[62,63] One advantage of the European Influenza Surveillance Scheme is that RSV detections can also be reported in addition to influenza.

Surveillance of respiratory infections can provide longitudinal data and can be used to help measure the impact of vaccines that decrease the burden of acute respiratory illness. For this purpose a vaccine surveillance network has been established in the United States for children from 0 to 4 years of age. Although there are currently no licensed RSV vaccines, effectiveness of RSV monoclonal antibody prophylaxis in high risk children could be evaluated with the data collected by this network.[64]

Influenza virus and RSV infection are clinically indistinguishable and a substantial proportion of patients diagnosed with influenza-like illness are infected by RSV[65] and influenza is detected in children with suspected RSV infection.[66] Influenza causes winter epidemics and leads to hospitalisations and mortality.[17,67] Patients with influenza A are more likely to present with fever, whereas patients with RSV will likely present with respiratory complaints and wheezing.[66] Although RSV is not a notifiable disease in Europe it causes substantial morbidity in the population and is an important confounder in influenza surveillance, also because both RSV and influenza peak in wintertime. Therefore the report of RSV in addition to influenza is relevant. Surveillance data are increasingly used to monitor long-term trends or to make international comparisons.[59]

Diagnosis

Respiratory samples are obtained for diagnostic testing. There are several specimen types: throat swabs, nasal swabs, nasopharyngeal swabs, nasopharyngeal washes and nasopharyngeal aspirates. Conventional diagnostic methods include virus isolation, immunofluorescence and enzyme-linked immunosorbent assays that detect antigen. Viral culture in combination with immunofluorescence has been considered the “gold standard” for laboratory diagnosis. The last decade molecular techniques are increasingly used for the

detection of respiratory pathogens and have become the "new gold standard" for the diagnosis in respiratory virus infections.[68] Multiplex real-time PCR methods are more rapid and sensitive compared to the conventional methods.[69,70]

The application of real-time PCR increases the sensitivity for respiratory viral diagnosis and results can be obtained within 6 hours, thus increasing clinical relevance.[71] Rapid laboratory diagnostic investigation clearly is a prerequisite for effective antiviral treatment. Additionally, the laboratory testing is important to reduce the use of antibiotic in case of an uncomplicated viral infection.

Children are known to shed RSV in high titres for up to several weeks, whereas shedding in adults and the elderly is presumed to be of relatively low titre and short duration.[72] As a consequence conventional methods that are suitable for diagnosis in children lacks sensitivity in older patients.[73] To overcome this lack of sensitivity real-time PCR methods can be used. For the frail elderly rapid antigen detection methods were found to be insensitive.[73] Viral shedding may have occurred before the patients seek care. Moreover, clinicians have usually low suspicion for RSV infection in adult patients and this results in the infrequent use of diagnostic tests.[10] In addition to the elderly, real-time PCR is also a sensitive method for the rapid diagnosis of RSV in immuno-compromised adults and represents a significant improvement over existing virus detection methods for this patient group.[74]

Aim and relevance

The aim of this thesis was to improve surveillance and diagnostic methods of respiratory syncytial virus (RSV) in Europe. We have investigated:

a. which countries collect RSV data and whether these data are being reported timely,
b. the occurrence of RSV and the relation with meteorological factors,
c. what diagnostic methods are used to detect RSV, and
d. what factors relate to the sensitivity of a diagnostic RSV test.

With respiratory virus surveillance one can get insight into the occurrence of winter infections, what respiratory pathogen causes the illness, when is the peak and what is the effect of the winter infections on the population.

Respiratory tract infections are common. It is important to have a reliable report on the incidence of respiratory infections, preferably by age group, and the laboratory testing to identify the cause of respiratory infections. This information will provide directions for possible target groups for the development of vaccines, such as for RSV and para-influenza virus.

Outline of this thesis

Chapter 2 describes surveillance methods used in influenza reporting surveillance systems in Europe and investigates whether testing is performed for other respiratory viruses, including RSV. In Chapter 3 recommendations for surveillance of RSV are made and in Chapter 4 the progress over seven years in RSV surveillance is presented. In Chapter 5 the variation of respiratory syncytial virus and the relation with meteorological factors is presented. Chapter 6 and 7 focus on the diagnostic methods; in Chapter 6 the impact of laboratory characteristics on the molecular detection of respiratory syncytial virus is investigated and in Chapter 7 we investigate the sensitivity of nasopharyngeal aspirates and swabs with real-time polymerase chain reaction for the main respiratory pathogens of childhood. Finally, in Chapter 8, the results of the earlier chapters are summarised and discussed and the implications and recommendations for future research are formulated.

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2

Methods for sentinel virological surveillance of influenza in Europe - an 18-country survey

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Abstract

The European Influenza Surveillance Scheme (EISS) is based on an integrated clinical and virological surveillance model. To assess the comparability of virological data, a questionnaire was sent to participants in June 2002 enquiring about specimen collection, laboratory diagnosis of influenza and tests for other respiratory infections. The results showed differences, but also uniformity in virological data collection methods. Similarities were reported for the specimen collection procedures; the type of swab and the transport conditions were comparable. The diagnostic methods were diverse; differences were seen in the (sub)typing methods, with PCR used most often in western countries. The findings will be helpful for the interpretation of virological data collected by sentinel physicians and for the creation of a Community Network of Reference Laboratories for Human Influenza in Europe. Important objectives of the Community Network include the harmonisation of virological methods and the application of quality assurance assessments for the national reference laboratories.

Introduction

Influenza is well recognised as an infectious disease that causes considerable morbidity and mortality in the human population.[1,2] In addition, there is the ever-present threat of an influenza pandemic.[3] In Europe, national influenza surveillance networks have been established since the 1950s. In the late 1980s, efforts were made to improve surveillance by integrating data on a European level through a number of collaborative projects that led to the European Influenza Surveillance Scheme (EISS). The basis of the scheme is combined clinical and virological surveillance of influenza in the general population. Sentinel physicians report cases of influenza-like illness (ILI) or acute respiratory infection (ARI) to a national data collection centre and obtain respiratory specimens from patients for laboratory testing.[4,5]

With regard to the surveillance of infectious diseases such as influenza, the role of the European Union (EU) has become more important in recent years.[6] The surveillance of influenza is a key element of the European influenza pandemic preparedness plan. An important task of surveillance is the early detection of influenza and the characterisation of potential pandemic strains

from clinical specimens.[7] To improve influenza surveillance in Europe, the EU has supported the creation of a Community Network of Reference Laboratories for Human Influenza[7] to accomplish several tasks, including the co-ordination of methods employed by the Member States for the diagnosis of influenza.

The European Scientific Working group on Influenza conducted an inventory in 1996 on the laboratory diagnostic and surveillance methods in 24 European countries.[8] This study showed that the techniques used in influenza surveillance were heterogeneous and the performance of virological surveillance was therefore difficult to compare between countries. The methods used for the virological surveillance of influenza may have changed since 1996 and we wanted to have an update of the methods currently used for the testing of sentinel respiratory specimens in Europe. In addition, we wanted to know whether tests were routinely performed for the detection of other respiratory pathogens besides influenza. The inventory aimed to determine the status of virological methods routinely used by sentinel influenza surveillance networks participating in EISS during the 2001-2002 season.

Material and Methods

A questionnaire on virological methods used for influenza diagnosis and surveillance was developed and sent electronically to all EISS collaborating surveillance networks in June 2002. People that were responsible for collecting virological data in each network were asked to complete the questionnaire. If a network had more than one reference laboratory, respondents were asked to complete a single questionnaire. Twenty-one networks participated in the study.

The following topics were included in the questionnaire: specimen collection, laboratory diagnosis of influenza and tests for other respiratory infections in addition to influenza. The questions in the survey concerned data collected during the 2001-2002 influenza season. All 21 networks completed the questionnaire. Results based on sentinel data are presented for all networks except for Poland and Sweden. The results from Poland and Sweden are based on data from non-sentinel sources.

Table 2.1: Sentinel specimen collection and transport*

Network	Material collected by sentinel pysicians	Transport medium	Mode of Transport	Temperature at transport[1]	Delay of transport (hrs)
Belgium	Two nasal and one throat swab	EMEM containing antibiotics + fungizone	Mail	Ambient	24-48
Czech Republic	Nasopharyngeal swabs, blood[2]	Special viral transport medium (NIC)	Ambulance	4 °C	24
Denmark	Nasal swabs and aspirates	Viral transport medium	Mail	Ambient	24-72
England	Nasal and throat swabs	NK	Mail, courier	Ambient	48-120[3]
France	Nasal or throat swabs	Medium containing penicillin, streptomycin and amphotericine B	Mail	Ambient	24-72
Germany	Throat swabs	Virocult	Mail	Ambient[4]	24-72[5]
Ireland	Combined nasal and throat swabs	Viral transport medium	Mail	NK	48
Italy	Throat swabs	Virocult (MedicalWire, England)	Mail, courier	Ambient	24-120[5]
Netherlands	Nasal and throat swab	GLY-medium+pimaricine	Mail	Ambient	24-48
Northern Ireland	Nasal and/or throat swabs	PBS+pencicillin, streptomycin and amphotericin	Mail, special delivery	Ambient	24
Norway	Nasopharyngeal swabs	Hanks's Balanced salt solution containing bovine albumin, fungizone and penicillin	Mail	Ambient	24(-48)
Poland	Nasal and throat swabs	Sterile PBS and antibiotics	Courier	8ºC	24-48
Portugal	Nasal swabs	Virocult (MedicalWire MW 950/974/975)	Express mail	Ambient	18-24
Romania	Nasal and throat swabs	Tryptose phosphate broth with gelatine	Mail, courier	4ºC	24-72
Scotland	Nasal and throat swabs	Guanidine based viral Lysis buffer	Mail	NK	24-48
Slovak Republic	Nasal and throat swabs (blood)	Hanks's Balanced salt solution or viruculture medium with BSA, antibiotics and antimycotics	Courier	4ºC[6]	0-72
Slovenia	Nasal and throat swabs	GIBCO EMEM medium	Mail, courier	Ambient[7] 4ºC[8]	1-48
Spain	Nasal and throat swabs	Saline solution +antibiotics	Courier	4ºC[6]	24
Switzerland	Combined nasal and throat swabs	GLY-medium + antibiotics	Mail	Ambient	24-48
Wales	Nasal and throat swabs (blood)	Medium containing minimal essential salts buffer and indicator solution	Mail	Ambient	24-72

Abbreviations: NK = Not known; BSA = bovine serum albumin; EMEM = eagles minimum essential medium; GLY = glucose lactalbumin yeast; PBS = phosphate buffered saline, NIC = National Influenza Centre.

* Sweden was not included in the table as it did not collect sentinel specimens and the techniques used at the 24 laboratories performing influenza detection are too varied to be included in the table.

1 Ambient means no control of temperature.
2 Not regular.
3 Majority arrives in 48 hours.
4 Storage swab containments at ambient temperature, swabs kept at 4ºC.
5 Majority arrives in 24 hours.
6 If possible.
7 When transported by mail.
8 When transported by courier.

Results

Sentinel specimen collection and transport
Information on specimen collection is presented in Table 2.1. Most networks (12/20) collect nasal as well as throat swabs. The remaining networks collect either nasopharyngeal, or nasal, or throat swabs. In addition, three networks collect blood samples and one network nasal aspirates. Transport of the swabs occurred by mail in 16 networks and by courier in seven networks. Some networks used special delivery (Northern Ireland) or ambulance (the Czech Republic) for the transport of the swabs. The temperature at transport was ambient in 13 networks and 4ºC in five networks. The viral transport medium meant to preserve virus viability used was diverse, but usually contained antibiotics to inhibit growth of other microorganisms. Scotland used a lysis buffer specifically developed for preservation of nucleic acid, and therefore only suitable for PCR. The time delay in transport of the material from the sentinel physician to the laboratory varied between 0-120 hours for all networks; most networks reported a delay of 24-48 hours.

Methods used for sentinel virological surveillance
The methods routinely used by the EISS networks to isolate or identify the influenza viruses in sentinel respiratory specimens are presented in Table 2.2.

All but two networks (the Netherlands and Scotland) used culture on MDCK cells for the detection of influenza viruses. Seven networks used culture on embryonated chicken eggs, and five networks used other cell lines in addition to MDCK cells. Diverse rapid techniques for virus detection are used, with RT-PCR most often used in the western countries and ELISA in the eastern countries.

The delay between specimen collection and the test result for typing (determination of influenza A or B) and subtyping (determination of H subtype and occasionally the N subtype) is shown in Table 2.2. The delay was variable and differed between EISS networks. A comparison of the delay in typing and subtyping is difficult to make since a variety of methods were applied to determine the type and subtype. For example, by using subtype specific PCR assays typing and subtyping can be done directly on the clinical specimen, whereas when typing and subtyping a virus isolate, the time needed to grow the virus is the defining factor.

Table 2.2: Laboratory methods in Europe used for sentinel surveillance of influenza

Network	Virus detection		Typing (A/B)	Typing delay (days)[6]	Subtyping (H and/or N)[7]	Subtyping delay (days)[6]
	Virus isolation (culture)	Rapid tests				
West						
Belgium	MDCK	Directigen Flu A+B	ELISA	1-3	PCR	14-21
Denmark	MDCK	ELISA, PCR	ELISA, PCR	5-10	HAI	14-21
England	MDCK, MK	PCR	HAI, ELISA, PCR, IF	1-2	HAI, PCR	1-2
France	MDCK	ELISA	HAI, ELISA	NK	HAI, PCR[1]	NK
Germany	MDCK	PCR	PCR	NK	HAI[2], PCR[2]	NK
Ireland	MDCK	PCR	PCR	2-7	PCR	2-14
Italy	MDCK, CE	PCR, dIF	HAI, PCR	2-10	HAI, PCR	2-10
Netherlands	MK	PCR	HAI	4-10	HAI	5-11
Northern Ireland	MDCK, CE	dIF	IF	1-2	PCR	1-3
Norway	MDCK	PCR	PCR, IF, HAI	2-7	PCR, IF, HAI	2-7
Portugal	MDCK, CE	PCR	PCR	3-4	PCR	3-4
Scotland	Not done	PCR	PCR	2-10	Not done	14-28
Spain	MDCK, HEp-2 + human lung fibroblast[3]	PCR	PCR	2-5	PCR, HAI	3-10
Sweden	MDCK	Not done	PCR, IF, HAI	NK	PCR, IF, HAI	NK
Switzerland	MDCK, LLC-MK2, A549	Not done	IF	7-9	PCR, HAI	NK
Wales	MDCK, MK	dIF	IF	1-14	Not done	14-100
East						
Czech Republic	MDCK, CE	ELISA IPT[4]	ELISA, IPT	1	HAI	3-12
Poland*	MDCK, CE[5]	dIF	HAI, IF	1-14	HAI	1-14
Romania	MDCK, CE	ELISA	HAI, ELISA	1-2	HAI, NI	2-6
Slovak Republic	MDCK, CE	ELISA, Directigen Flu A+B	HAI, ELISA	2-5	HAI	3-6
Slovenia	MDCK, MK	ELISA, PCR	PCR, IF	1-8	HAI	NK

Abbreviations: NK = Not known; MDCK = Madin-Darby canine kidney; MK = monkey-kidney; CE = chicken egg; ELISA = enzyme-linked immunosorbent assay; PCR = polymerase chain reaction; HAI = haemagglutination inhibition; (d)IF = (direct) immunofluorescence; NI = neuraminidase inhibition; A549 = human lung cancer cell line.

* Data for Poland and Sweden are from non-sentinel surveillance systems. The Swedish responses to the typing procedures concern cultivated specimens sent by six virological laboratories to the Swedish Institute Desease Control.

1 PCR is used for H1N2 subtyping.
2 Method differs by laboratory.
3 The HEp-2 cell line is an epithelial cancer cell line.
4 Immunoperoxidase staining.
5 For the 2002-2003 influenza season MDCK cells will be mainly used.
6 Time between specimen collection and typing or subtyping.
7 Networks were not asked to specify the methods used for subtyping H and N separately.

For typing of influenza viruses the following methods were applied: PCR (11 networks), HAI (9 networks), IF (8 networks) and ELISA (7 networks). For subtyping the HAI assay was used in 15 networks. However, PCR was also used for subtyping in twelve networks. A total of nine networks applied more than one test to subtype influenza viruses. None of the five networks in eastern Europe used PCR, while 12 out of 14 networks that perform subtyping in western Europe used PCR (Table 2.2). Of these, eight networks used both HAI and PCR.

Testing sentinel specimens for other respiratory infections

Thirteen out of nineteen networks (the Czech Republic, England, France, Germany, the Netherlands, Northern Ireland, Portugal, Romania, Scotland, Slovenia, Spain, Switzerland, Wales) reported that they collect information on respiratory pathogens other than influenza virus in sentinel respiratory specimens. All thirteen networks collected information on respiratory syncytial virus (RSV), six networks collected data on adenovirus, five networks collected data on parainfluenzavirus and three networks collected data on rhinovirus. Three networks (England, the Netherlands and Slovenia) had information on other respiratory pathogens (e.g. coronavirus, Chlamydia pneumoniae, human metapneumovirus) (data not shown). Eleven networks reported that the sentinel swabs were tested for both influenza virus and RSV.

Discussion

The results highlight similarities in the specimen collection and transport procedures in the EISS networks. In most networks nose swabs as well as throat swabs were obtained and transported by mail to the laboratory. The laboratory methods used were heterogeneous, which confirms earlier findings.[8] For virus culture, most networks used the same type of cells (MDCK), but for typing and subtyping of influenza viruses different methods (ELISA, HAI, PCR) were used. ELISA was more often used for typing and subtyping in eastern Europe and PCR was more frequently used in western Europe. Another important finding is that the majority of networks in EISS reported that they test sentinel swabs for other viruses (in particular RSV).

The type of respiratory specimen, the delay in the transport of swabs, the transport medium and the transport temperature are important factors that could potentially lead to an underestimation of the number of laboratory confirmed clinical cases of influenza reported by sentinel physicians. Our study has shown that most EISS networks used nose and/or throat swabs. In general, these are considered to be the right specimens for techniques such as culture and immunofluorescence.[9] The transport of samples is advised at 4ºC or frozen at -70ºC.[9] The outcome of our survey is that the specimens were often sent by post, at an ambient temperature and usually took 24-48 hours to reach the laboratory. This can be considered suboptimal, especially for virus culture. However, a study carried out in England and Wales found that clinical specimens sent by post provided good results when using multiplex RT-PCR techniques, although it is likely that there is some degradation of viral nucleic acid when specimens are transported this way.[10] Another factor, the viral transport medium, should ideally include a balanced salt solution at neutral pH with protein stabilizers such as gelatine or bovine serum albumin (BSA) and antibiotics.[9] The EISS networks used diverse media for the transportation of specimens, but in general these media met the mentioned demands.

All but one network used virus isolation on cell culture as the primary method for the detection of influenza virus. This approach is commonly used as the EISS laboratories characterise their virus isolates and/or send material to the WHO Collaborating Centre at Mill Hill for strain characterisation, an activity that is very important to map the spread of influenza globally and to establish the influenza vaccines in the southern and northern hemispheres each season. The reasons for using additional techniques, like PCR and ELISA, for detection were confirmation of the results, increased sensitivity and the detection of other respiratory pathogens such as adenovirus (e.g. in Slovenia, Spain and Switzerland).

The harmonisation of virological testing methods is an important objective of EISS. To initiate these efforts, a first Quality Control Assessment (QCA) was performed during the 2000-2001 season.[11] Differences in virological results can be associated with the use of different laboratory techniques (e.g. PCR vs. cell culture[10,12,13]) or differences in the application of the same laboratory technique (e.g. PCR). The first QCA, carried out in 16 EISS laboratories, found that the sensitivity of the RT-PCR in Europe varied widely (40-100% for influenza, 71-86% for RSV), depending on the laboratory.[11] A second QCA was

carried out during the 2002-2003 season and considerable improvements in the sensitivity rates were found (data not shown). The results of the first two QCAs, and QCAs planned in the future, will be used to further harmonise virological testing methods in EISS.

The finding that sentinel specimens were being tested for other respiratory infections is important for EISS, as many agents are associated with clinical symptoms of influenza-like illness and acute respiratory infection. An important pathogen that contributes to this burden of disease is RSV; in terms of mortality the role of RSV is suggested to be even greater than influenza B and influenza A/H1N1.[2] The results of the inventory indicated that a large proportion of the networks tested sentinel specimens for RSV and we could therefore collect more detailed information on RSV activity in Europe. These findings have led to the creation of an RSV Task Group to explore how the surveillance of RSV could be better developed and further integrated into EISS.

In conclusion, sample collection and shipment are more or less similar whereas detection and (sub)typing methods are heterogeneous among the EISS networks. Despite this heterogeneity, results for detection and (sub)typing can be considerably improved when carefully controlled by external quality control, as the results of the two QCA studies showed. Further improvements may be made by a better harmonization and standardization of the applied methods. EISS will therefore take a number of actions within the framework of the recently created Community Network of Reference Laboratories for Human Influenza; these include the definition of basic tasks to be carried out by the laboratories, the preparation of standardised laboratory protocols and further QCAs.

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3

Surveillance recommendations based on an exploratory analysis of respiratory syncytial virus reports derived from the European Influenza Surveillance System

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Abstract

Background
Respiratory syncytial virus (RSV) is an important pathogen that can cause severe illness in infants and young children. In this study, we assessed whether data on RSV collected by the European Influenza Surveillance Scheme (EISS) could be used to build an RSV surveillance system in Europe.

Methods
Influenza and RSV data for the 2002-2003-winter were analysed for England, France, the Netherlands and Scotland. Data from sentinel physician networks and other sources, mainly hospitals, were collected. Respiratory specimens were tested for influenza and RSV mainly by virus culture and polymerase chain reaction amplification.

Results
Data on RSV were entered timely into the EISS database. RSV contributed noticeably to influenza-like illness: in England sentinel RSV detections were common in all age groups, but particularly in young children with 20 (40.8%) of the total number of sentinel swabs testing positive for RSV. Scotland and France also reported the highest percentages of RSV detections in the 0-4 year age group, respectively 10.3% (N=29) and 12.2% (N=426). In the Netherlands, RSV was detected in one person aged over 65 years.

Conclusions
We recommend that respiratory specimens collected in influenza surveillance are also tested systematically for RSV and emphasize the use of both community derived data and data from hospitals for RSV surveillance. RSV data from the EISS have been entered in a timely manner and we consider that the EISS model can be used to develop an RSV surveillance system equivalent to the influenza surveillance in Europe.

Background

Respiratory syncytial virus (RSV) is the most important viral agent causing severe respiratory disease in infants and young children.[1] Although infrequently recognised, RSV infection is common in adults and sometimes causes severe illness especially in the elderly.[2,3] RSV infection presents with similar clinical symptoms to other respiratory viral infections, including influenza.[4,5] Influenza is associated with increased general practice consultation rates[6], increased hospital admissions[7] and excess deaths. [7,8] RSV and influenza viruses frequently co-circulate around the same time of the year making it difficult to estimate their separate clinical impacts.[9] The contribution of RSV to influenza-like illness needs to be assessed if this is to be used as a clinical endpoint for evaluating influenza vaccine effectiveness.[10,11]

Advances in the development of RSV vaccines[12] has prompted a need for research into the societal and economic impact of RSV infection in order to make sensible decisions about their potential use. So far, prevention of severe RSV-associated bronchiolitis has only been achieved in high-risk infants by passive administration of the humanized monoclonal antibody palivizumab. [13] A timely RSV surveillance system could be valuable in optimizing the use of palivizumab increasing its efficiency and reducing costs[14] as doctors would become aware of the circulation of the virus and probable cause of illness in high-risk infants.

Monitoring influenza activity has been coordinated by the European Influenza Surveillance Scheme (EISS) since 1996. EISS is one of the Designated Surveillance Networks established to monitor infectious diseases in the European Union.[15] The surveillance is performed by sentinel primary care physicians and is based on an integrated clinical and virological surveillance model.[16,17] In addition to the sentinel surveillance results on specimens obtained from other sources (mostly hospitals) are also reported. Currently, no integrated European surveillance such as the EISS is in place for RSV, although RSV surveillance initiatives have been reported by several EU Member States (Germany, the Netherlands, France, United Kingdom).

We aimed to assess whether data already collected within EISS could be used to build an RSV surveillance system in Europe. We consider timeliness of RSV reports to EISS as well as the collection of both sentinel and hospital-based RSV

data by age group important for RSV surveillance. We analysed RSV and influenza virus reports in different age groups and study populations in four European countries, and we assessed whether RSV and influenza data were reported in a timely manner into the EISS database.

Methods

Influenza and RSV data available in the EISS database for the 2002-2003 winter (weeks 40/2002 to 20/2003) were analysed. Data from both sentinel practitioners and other sources (from hospitals, non-sentinel physicians, residential institutions) were used. Data from these other sources are referred to as non-sentinel in this paper. Four countries were included: England, France, the Netherlands and Scotland. Data for France was confined to nine regions in the south covering 37.5% of the French population. The selection of countries was based on the availability of both sentinel and non-sentinel virological data on RSV and influenza, and on a minimum number of 500 non-sentinel respiratory specimens tested for RSV and/or influenza during the study winter.

Specimen collection and analysis

Combined nose and/or throat swabs were obtained from selected patients presenting to physicians in sentinel practices with influenza-like illness. In addition, general practitioners in Scotland were requested to sample patients with acute respiratory infections in the absence of patients presenting with influenza-like illness. The respiratory specimens were transported to participating laboratories mainly by regular mail.[18] Similar laboratory methods were used in three out of four countries (Table 3.1); France used enzyme-linked assays including ELISA (enzyme-linked immunosorbent assay) instead of RT-PCR (reverse transcriptase polymerase chain reaction).[19] Although the sensitivity of ELISA has been reported to be lower than RT-PCR, ELISA is reliable for rapid laboratory diagnosis of influenza in infants and young children; for older patients application of virus isolation or RT-PCR is necessary.[20] Samples were defined positive for RSV or influenza when at least one laboratory test yielded a positive result.

Table 3.1: Laboratory methods used for RSV and influenza virus detection or isolation

	Methods used for RSV detection/isolation	Methods used for influenza virus detection/isolation
England	RT-PCR, culture	RT-PCR, culture
France	ELFA, culture	ELISA, culture
Netherlands	RT-PCR, culture	IF, RT-PCR, culture
Scotland	RT-PCR (multiplex)	RT-PCR (multiplex)

ELFA: enzyme-linked fluorescent assay (automated qualitative test)
ELISA: enzyme-linked immunosorbent assay
IF: immunofluorescence; this technique was not performed for sentinel samples
RT-PCR: reverse transcriptase polymerase chain reaction

The sentinel networks in England and Scotland did not apply a precise case definition for influenza-like illness. The case definition used in France was: sudden onset of respiratory symptoms with infection context (fever, headaches), in the absence of other diagnosis. The case definition in the Netherlands contained the following criteria: an acute onset of illness (prodromal stage $\leq$ 3-4 days), and at least one of the symptoms: coughing, rhinitis, sore throat, frontal headache, retrosternal pain, or myalgia.[21] The selection of patients for swabbing was not based on pre-established diagnostic criteria. In France many samples were obtained from children because paediatricians as well as general practitioners are included in the surveillance network.[22] Virological test results from sentinel practices were specified by age group (0-4, 5-14, 15-64, and over 65 years). Non-sentinel specimens obtained from hospitals were mostly examined for either RSV or influenza and not both viruses.

Data analysis

We examined the timeliness of RSV data entry into the EISS database by investigating whether data on RSV were included in the EISS Weekly Electronic Bulletin and compared this to the timeliness of influenza data. The Weekly Electronic Bulletin is published on the EISS website each Friday and reports the influenza activity for EISS member countries collected during the previous week. More details on the Weekly Electronic Bulletin can be found in the technical note.[23] For the statistical analysis, the comparisons of percentages were performed using EpiTable in Epi Info version 6.04d (January 2001). Statistical significance was concluded if the p-value was < 0.05.

Results

Respiratory Syncytial Virus

RSV detections are summarized for each of the four countries in Table 3.2. For England RSV detections from sentinel practices were common in all age groups, but especially in young children aged 0-4 years with 40.8% (N=49) testing positive for RSV. The highest percentage RSV positive specimens was reported for the 0-4 age group in Scotland (10.3%, N=29) and France (12.2%, N=426) as well. In the Netherlands, RSV was detected in one person aged over 65 years. In England, the percentage of RSV positive reports (26.7%) was higher than that for influenza (21.3%; Chi^2=3.9, p=0.048). Non-sentinel data (available by age group for England and Scotland only) showed that 92% or more of the RSV positive reports were obtained in children 0 to 4 years.

Influenza

Influenza virus detections are summarized in Table 3.3. Sentinel data indicated more influenza reports than RSV in Scotland, France and the Netherlands. The highest specimen positive proportions of influenza viruses were reported in children aged 5-14 years (England 52.4%; France 41.6%; Scotland 23.9%). Non-sentinel data (available by age group for England and Scotland only) showed most confirmed influenza cases in the 0-4 and 15-64 age groups.

Timeliness

In each of the four countries sentinel and/or non-sentinel RSV data were entered in a timely manner, within 1-2 weeks after specimen collection, into the EISS database. A total of 26 Weekly Electronic Bulletins were published during the 2002-2003 winter season, from week 42 to week 15 of the following year. For the Netherlands, timely RSV data were not available for weeks 42-50 because data entry only started that season. For the four countries data on influenza was reported for a total of 97 out of 104 weeks and RSV reports where made in a timely manner in 87 out of 104 weeks.

Table 3.2: RSV detections by country and age group for sentinel and non-sentinel specimens.

Country	Sentinel			Non-sentinel
	Total number of specimens tested	Number of specimens tested positive for RSV	Percentage positive (%)	Number of specimens tested positive for RSV
England				
0-4 year	49	20	40.8	3,982
5-14 year	63	16	25.4	13
15-64 year	307	77	25.1	60
> 65 year	45	13	28.9	11
NK	11	1	9.1	85
Total	475	127	26.7	4,151
Scotland				
0-4 year	29	3	10.3	1,474
5-14 year	67	0	-	24
15-64 year	444	13	2.9	56
> 65 year	58	3	5.1	19
NK				15
Total	598	19	3.2	1,588
France				
0-4 year	426	52	12.2	
5-14 year	442	20	4.5	
15-64 year	557	14	2.5	
> 65 year	32	0	-	
Total	1,457	86	5.9	1,748
Netherlands				
0-4 year	0	-	-	
5-14 year	7	0	-	
15-64 year	42	0	-	
> 65 year	7	1	14.3	
Total	56	1	1.8	1,757

NK: Not known.

Table 3.3: Influenza virus detections by country and age group for sentinel and non-sentinel specimens

Country	Sentinel			Non-sentinel
	Total number of specimens tested	Number of specimens tested positive for RSV	Percentage positive (%)	Number of specimens tested positive for RSV
England				
0-4 year	49	12	24.5	260
5-14 year	63	33	52.4	81
15-64 year	307	51	16.6	143
> 65 year	45	2	4.4	45
NK	11	3	27.3	12
Total	475	101	21.3	541
Scotland				
0-4 year	29	0	0	64
5-14 year	67	16	23.9	53
15-64 year	444	13	2.9	108
> 65 year	58	2	3.4	31
NK				1
Total	598	31	5.2	257
France				
0-4 year	426	82	19.2	
5-14 year	442	184	41.6	
15-64 year	557	109	19.6	
> 65 year	32	1	3.1	
Total	1,457	376	25.8	243
Netherlands				
0-4 year	0	0		
5-14 year	7	3	42.9	
15-64 year	42	8	19.0	
> 65 year	7	4	57.1	
Total	56	15	26.8	239

NK: Not known.

Discussion

We have assessed whether EISS could be used to build a European RSV surveillance system. Surveillance systems must be timely in order to be effective. The EISS system has demonstrated timeliness in providing data on influenza and as this report shows in four countries, timely data on RSV. Sentinel data indicated that RSV contributed considerably to influenza-like illness, especially in young children. Since the infrastructure of EISS is well established[24], we suggest the use of EISS as a model for setting up an RSV surveillance system in Europe.

Healthcare based surveillance systems are dependent upon persons consulting doctors. For common respiratory infections, there are many more infected persons in the community who do not consult their doctor. Selection biases which start with the decision to consult are compounded at the point of consultation. In addition, sensible use of virological investigation does not necessarily mean that every suspect case is investigated. Certainly as far as patients in the community are concerned, routine virological investigation for a common condition which is usually minor is not economically justifiable. Furthermore, the patient's willingness to be sampled will always be a major consideration.

The EISS differentiates between sentinel and non-sentinel sources of data. Sentinel networks in Europe are chiefly based on general practices (and in some European countries also on paediatric primary care services) and these are essential to provide insight into what is happening in the community at large. However, the hospital admission is a useful proxy for severity of illness and it is desirable therefore to have access to additional hospital source data. This is particularly important when an illness is common in all age groups but hospital admission is much more likely in particular age groups. Accordingly we wish to encourage data collection from hospitals either on a routine basis from all hospitals or perhaps more thought might be given to the development of sentinel hospitals with a higher level of commitment to high quality data capture and more structured virological investigation.

Our study has shown that the age distribution of RSV positive cases was similar in the four countries. For England relatively more RSV than influenza was reported but this was not so in the other three countries. A possible reason for

this could be the use of a more sensitive diagnostic test in England compared to the other countries. Within EISS the need for harmonization of laboratory methods is recognised and a Community Network of Reference Laboratories has been established in 2003. This Network encourages the harmonisation of laboratory methods for the detection of influenza in EISS and assesses the quality of laboratory testing for influenza and RSV.[25]

To see whether the data for England were consistent with earlier findings, we compared our results to data published previously on RSV.[10] More RSV than influenza virus was reported for one of the winter seasons (1997-98), this finding is similar to what we have reported for 2002-2003. It is important to note that differences between countries and seasons can simply be due to seasonal variation; lower proportions of RSV detections from patients with influenza-like illness have been observed for England as well.[26]

The sentinel networks in all four countries used combined nose and/or throat swabs inserted in the same vial. These have proved reliable for influenza surveillance.[27] However, the best site to collect material for viral detection may differ between influenza virus and RSV. Nasal swabs may be less specific than nasopharyngeal aspiration[28], on the other hand swabs are probably less painful and easier to obtain in a general practice setting. Facilities for sampling patients in the hospital are generally better than those in the community since there may be increased opportunity for sample collection and less limitation on sample transportation with hospitals linked directly to microbiology laboratories.

The diagnosis made, the selection of patients for swabbing, the quality of the swab taken, the transport procedures, the virological investigation methods and the experience of the laboratory concerned, all influence virus detection rates. The majority of sentinel respiratory specimens did not test positive for either influenza or RSV. This may be explained by other respiratory viruses that are known to cause symptoms similar to influenza and RSV infection[29,30] but few are regularly investigated. As an example, for Scotland, 83 (13.9%) sentinel swabs tested positive for picornavirus during the 2002-2003 winter season. Furthermore, positivity rates differed considerably between countries: e.g. in Scotland the percentage positive for RSV and influenza was only 8%. In the future, the EISS might implement more respiratory viruses for surveillance purposes simultaneously after introducing RSV. Nine countries in EISS already

tested sentinel specimens for more viruses than RSV and influenza in 2002, e.g. for human metapneumovirus, rhinovirus, coronavirus, adenovirus, C. pneumoniae or para-influenza virus.

Discrepancies in positivity rates could reflect several factors mentioned above; but it is also possible that payment to general practitioners for taking swabs in Scotland leads to sampling bias. In addition, general practitioners in Scotland are requested to take samples from patients with acute respiratory infections in the absence of influenza. Relatively few respiratory specimens were collected by the Dutch sentinel network which can lead to underestimation of the incidence of RSV and influenza as judged from virological data. This seems in particular true for children and the elderly.

The current methodological differences between countries and the constraints of the study (data for four countries and one season) impose limitations. Since we selected and analysed data for the four countries that tested sentinel specimens during the 2002-2003 season for RSV, we cannot state that all members of EISS are able to comply with routine RSV reporting. However, this study demonstrated that it is possible to report RSV in addition to influenza. We believe our results pave the way for the development of an RSV surveillance system running in parallel to influenza surveillance.

Conclusions

Our conclusions relate to recommendations for an RSV surveillance programme.

1. Specimens collected as part of an influenza surveillance programme should also be tested for RSV.
2. Both combined nose/throat swabs and nasal pharyngeal aspirates are acceptable for RSV diagnosis.
3. The application of molecular techniques such as real time PCR in the diagnosis of respiratory disease has been demonstrated and we advocate this technique for RSV detection.
4. We encourage further developments on the use of standardized methods and laboratory techniques.
5. The development of a sentinel approach of representative hospitals should be considered.

6. We recommend the new networks joining EISS to integrate RSV surveillance alongside influenza.

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4

Progress in the surveillance of respiratory syncytial virus (RSV) in Europe: 2001-2008

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Abstract

Respiratory syncytial virus (RSV) surveillance is important to get insight into the burden of disease and epidemic pattern of RSV infection. This information is useful for healthcare resource allocation as well as the timing of preventive messages and palivizumab prophylaxis. For influenza surveillance the European Influenza Surveillance Scheme (EISS) was established in 1996, but no surveillance platform is available for RSV. To improve surveillance an RSV Task Group was established in 2003 and recommendations for RSV surveillance were developed. By 2008, progress was made for four out of six recommendations: the number of European countries testing specimens for RSV increased from six to fourteen; nose and/or throat swabs were generally used for detection of influenza and RSV; a total of 25 laboratories performed molecular testing for diagnosis and participated in a quality control assessment for RSV with an overall good performance; four of the ten countries that joined EISS in 2004 started reporting RSV detections in addition to influenza in the period 2004-8. Limited progress was achieved for standardising methods and the development of a sentinel surveillance system of representative hospitals. Improving RSV surveillance is possible by further harmonising the data collection and increased reporting of RSV.

Introduction

Respiratory syncytial virus (RSV) is the most important viral agent causing severe respiratory disease in young children.[1-3] RSV is also being recognised as a significant pathogen in adults[2,4]causing moderately severe respiratory disease especially in the elderly.[5,6] Influenza is widely recognised as a major cause of morbidity and mortality in humans.[7,8] Since RSV and influenza virus infections are associated with similar clinical symptoms[9] and frequently co-circulate around the same time of the year, there is substantial potential for confusion regarding the cause of influenza-like illness.[10]

Influenza and RSV account for similar numbers of deaths in children and their impact varies by winter and age group. RSV is associated with more deaths than influenza in children aged 1-12 months.[11] Excess deaths due to RSV and influenza virus infection have also been reported for the elderly population.[5,8] When comparing cause-specific mortality due to influenza virus and RSV

infection in all ages, it has been estimated that most deaths were associated with influenza A(H3N2) viruses, followed by RSV, influenza B, and influenza A(H1N1).[8]

While influenza is on the list of communicable diseases that must be covered by the European Community network for surveillance, RSV is not on this list.[12] Nonetheless, RSV causes considerable burden of disease and RSV surveillance is important for determining the burden of illness in all age groups and in defining seasonality and epidemic pattern. This facilitates the preparation of hospital settings to receive more children and to define the timing of the start of palivizumab prophylaxis.[13] Palivizumab can be administered as passive immunoprophylaxis and is the only strategy that has been demonstrated to reduce RSV hospitalisations in high-risk children.[14] For real-time influenza surveillance the European Influenza Surveillance Scheme (EISS), a collaborative multinational project, was established in 1996[15], but no such scheme was available for other respiratory viruses including RSV. Since RSV and influenza infections typically occur in the winter, EISS made it possible to report RSV detections into the EISS database, on a voluntary basis, from 1996 until September 2008.

In 2003 an RSV Task Group was established within EISS to explore the possibility to design a comprehensive RSV surveillance scheme within the EISS framework. This Task Group was composed of four epidemiologists and two virologists. Three meetings were organised between July 2003 and January 2006 and updates on the activities were presented to the EISS group during the EISS Annual Meetings. A retrospective analysis was carried out. Additionally, RSV surveillance recommendations were published in 2006[16], and are presented below:

1) Specimens collected as part of an influenza surveillance programme should also be tested for RSV.
2) Both combined nose/throat swabs and nasal pharyngeal aspirates are acceptable for RSV diagnosis.
3) The application of molecular techniques such as real time PCR in the diagnosis of respiratory disease has been demonstrated and we advocate this technique for RSV detection.
4) Further developments are encouraged on the use of standardised methods and laboratory techniques.

5) The development of a sentinel approach of representative hospitals should be considered.
6) New countries joining EISS are encouraged to integrate RSV surveillance alongside influenza surveillance.

Our objective was to assess whether the RSV reporting within EISS in the period 2004-2008 complied with these surveillance recommendations, and to describe the detection and reporting of seasonal influenza and RSV infections in six selected countries in Europe.

Methods

Data collection in EISS

EISS was based on an integrated clinical and virological surveillance model. Sentinel primary care physicians reported weekly the number of new cases of influenza-like illness and/or acute respiratory infections and obtained respiratory specimens from a sample of patients for laboratory testing. The specimens were tested for influenza and in several countries for RSV as well. Weekly consultation rates and laboratory test results were entered by the national surveillance networks into the EISS database via an internet-based system.[17] Non-sentinel, mainly hospital-based data for influenza and RSV were also collected, but will not be presented in this paper.

Since September 2008, European influenza surveillance has been carried out by the European Centre for Disease Prevention and Control (ECDC) and involves all 27 European Union Member states and Norway. Three other countries Serbia, Switzerland and Ukraine are reporting data to WHO Europe.

This paper presents a descriptive study. Surveillance data for seven winter seasons (2001-2 to 2007-8; week 40-20) in the EISS database were screened for RSV detections by country. The database containing virological detections of RSV and influenza was downloaded by September 2008. An RSV reporting country was defined as a country that reported at least 10 sentinel specimens positive for RSV from 2001-2008. With this method the progress for recommendation 1 and 6 could be assessed. For the other recommendations the progress was summarised by collecting relevant data from inventories and a quality control assessment.

RSV detections: six countries

Country selection

Data from the Czech Republic, France, Germany, Netherlands, Slovenia and the United Kingdom (UK) (represented by England and Scotland) were assessed to describe the RSV surveillance in these countries. All had reported data for at least five winter seasons. Sentinel primary care physicians included general practitioners (GPs) in the United Kingdom and the Netherlands, and GPs and paediatricians in the Czech Republic, France, and Germany, and GPs, paediatricians and specialists in Slovenia. The sentinel doctors represented 1-5% of all physicians working in the country.

Case definition

Data on new cases were based on reporting of consultations for influenza-like illness (ILI) in the Netherlands, Slovenia and United Kingdom. Consultations for acute respiratory infections (ARI) were collected in France and Germany. From 2001-2 to 2004-5 the Czech Republic reported the number of new cases of ARI, and from 2005-6 onwards they reported cases of ILI in addition to ARI.[18] Case definitions for ARI and ILI differed slightly between countries.[19] The type of specimen that was collected (nose and/or throat swab) as well as transport conditions were similar.[20] Samples were generally collected within five days after onset of symptoms and systematically tested for both influenza virus and RSV in all countries. In Germany, only specimens of children aged 0-3 years were tested for RSV. Cases were defined positive for RSV or influenza when at least one laboratory test yielded a positive result. Between-country comparisons will not be made due to methodological differences.

Results

Recommendation 1

Specimens collected as part of an influenza surveillance programme should also be tested for RSV.

Seventeen countries had reported RSV detections in the period 2001-2008: Austria, Croatia, Czech Republic, Denmark, Estonia, Finland, France, Germany, Italy, Luxembourg, Netherlands, Poland, Romania, Slovenia, Slovakia, Switzerland, UK- England and Scotland. Since England and Scotland have their own sentinel surveillance systems, these are presented separately in this

paper. The number of countries reporting influenza data increased from 18 in 2001-2 to 31 in the winter of 2007-8 (Table 4.1).

In 2001-2 only six countries reported RSV detections in addition to influenza, but their number gradually increased, particularly around 2003-4, among both countries that had participated since 2001 and new member states (see also results for recommendation 6). From 2005-6 no further increase in the number of countries reporting RSV was observed (Table 4.1).

Table 4.1: Reporting of RSV and influenza data to EISS in the period 2001-2008

Season	Countries reporting RSV*	Countries reporting influenza	Number RSV detections	Number influenza detections
2001-2002	6	18	203	2276
2002-2003	8	19	335	3787
2003-2004	12	22	143	2732
2004-2005	12	23	557	5483
2005-2006	14	28	803	3171
2006-2007	14	30	888	5077
2007-2008	13	31	929	5076

* 2001-2002: CZ, FR, DE, SI, CH, UK-E, UK-S.
2002-2003: CZ, FR, DE, NL, SK, SI, CH, UK-E, UK-S.
2003-2004: CZ, FR, DE, NL, SK, SI, CH, UK-E, UK-S.
2004-2005: AT, CZ, DK, FR, DE, IT, LU, PL, RO, SI, CH, UK-E, UK-S.
2005-2006: AT, CZ, DK, EE, FI, FR, DE, IT, LU, NL, PL, RO, SI, UK-E, UK-S.
2006-2007: AT, CZ, DK, EE, FI, FR, DE, IT, LU, NL, PL, RO, SI, UK-E, UK-S.
2007-2008: AT, HR, CZ, DM, EE, FI, FR, DE, LU, NL, PL, SI, UK-E, UK-S.

Abbreviations: Austria (AT), Croatia (HR), Czech Republic (CZ), Denmark (DK), Estonia (EE), Finland (FI), France (FR), Germany DE), Italy (IT), Luxembourg (LU), Netherlands (NL), Poland (PL), Romania (RO), Slovenia (SI), Slovakia (SK), Switzerland (CH), UK-England (UK-E), UK-Scotland (UK-S).

Recommendation 2
Both combined nose/throat swabs and nasal pharyngeal aspirates are acceptable for RSV diagnosis.

Different types of specimens are used for detection of influenza and RSV.[21] Generally the nasopharyngeal aspirates have a high sensitivity, and are often used in a hospital setting. Easier to use and less painful are nasal/nasopharyngeal swabs.[22] An inventory carried out in 2002 indicated that

in sentinel surveillance systems in Europe nose and/or throat swabs were taken.[20] Twelve out of 20 national networks collected combined nose/throat swabs. The remaining networks collected either nasopharyngeal, nasal, or throat swabs. In addition, three networks took blood samples and one network obtained nasal aspirates.[20] Since all countries had already used the recommended type of respiratory sample and fulfilled the recommendation, no progress was assessed after 2002.

Recommendation 3
The application of molecular techniques such as real time polymerase chain reaction (PCR) in the diagnosis of respiratory disease has been demonstrated and this technique is advocated for RSV detection.

In 2006, laboratories were invited to participate in a quality control study for molecular methods. Of the 33 laboratories participating in EISS, 25 performed this technique with an overall performance of 88% correct results.[23] The majority (22 out of 25) of laboratories used an in-house molecular assay. In particular, real time PCR and nested PCR assays provided the highest performance scores (93% correct score; range 70-100) and were used in 19 laboratories. Three laboratories used commercial assays and the percentage of correct results ranged from 50% to 80%.[23]

Recommendation 4
Further developments in the use of standardised methods and laboratory techniques are encouraged.

Limited progress was made in standardising methods. Only for influenza, not RSV, laboratory protocols were shared and standardised reagents were made available via the EISS website. However, with the application of molecular methods, as indicated in recommendation 3, and quality control assessment of this method, the quality of laboratory testing of RSV is ascertained.

Recommendation 5
The development of a sentinel system of representative hospitals should be considered.

No efforts were made to develop a European sentinel surveillance system consisting of representative hospitals, though national initiatives may have been undertaken. For example, a laboratory-based surveillance for RSV involving different hospital laboratories in Slovenia was implemented in 2006.[24]

Recommendation 6
We recommend the new networks joining EISS to integrate RSV surveillance alongside influenza.

Ten new countries became members of EISS between 2004 and 2008: Austria, Bulgaria, Croatia, Estonia, Finland, Cyprus, Greece, Hungary, Ukraine and Serbia.[25] Of these, four countries followed the recommendation and started reporting RSV data (Table 4.1).

RSV detections: six countries
To illustrate the data that were collected by EISS, we present the results of RSV detections for six countries. All countries reported at least five seasons of data, which provided insight in the occurrence of RSV in these countries. RSV and influenza detections are presented in Table 4.2. The percentage of RSV-positive specimens largely differed by season, e.g. from 3% to 19% in the Czech Republic (Table 4.2). For all seasons and countries together the percentage of RSV-positive specimens varied from 4% in Germany and the Netherlands to 16-18% in the United Kingdom. RSV activity usually started a few weeks before the onset of influenza activity (data not shown). The data collected are useful to describe the seasonality of RSV and show that RSV is detected in patients with ILI and/or ARI.

Table 4.2: Number of sentinel influenza and RSV detections by country in the period 2001-2008. The relative percentage of RSV cases is presented by the total number of tested samples

Country	RSV detections per season Mean (range)	Influenza detections per season Mean (range)	Total RSV and influenza detections Mean (range)	% RSV (range)
Czech Republic	18 (5-30)	206 (83-311)	223 (102-327)	8 (3-19)
France	145 (47-227)	1053 (824-1374)	1198 (947-1601)	12 (4-18)
Germany	43 (12-138)	1129 (553-2145)	1172 (568-2172)	4 (1-10)
Netherlands*	12 (1-19)	121 (15-142)	133 (16-153)	4 (0-16)
Slovenia	6 (1-12)	101 (69-132)	106 (77-135)	5 (1-12)
UK-England	44 (14-125)	231 (82-432)	275 (107-477)	16 (8-56)
UK-Scotland	23 (14-35)	101 (31-193)	123 (50-220)	18 (11-38)

* No RSV detections were reported for the Netherlands in the winters of 2001-2002 and 2004-2005.

Discussion and conclusion

Progress in RSV surveillance was made in the period 2001-2008, with the most obvious increase in the number of reporting countries during the time the RSV Task Group was active, between 2003-2006. Progress was made particularly in terms of the number of countries testing specimens for RSV and the use of molecular techniques. The results for the six countries that had reported at least five years of data showed that RSV surveillance and reporting is feasible in Europe. The overall percentage of RSV-positive specimens for the Czech Republic, France, Germany, Netherlands, Slovenia and the UK amounted to 4-18% indicating that a substantial number of patients who consulted their sentinel physician with influenza-like illness or acute respiratory infection actually had an RSV infection. The EISS surveillance is real time and therefore can be relevant for timing of the influenza and RSV peak and providing insight into the morbidity and seasonality of these respiratory illnesses.

Limited progress was made for recommendation 4 on the use of standardised laboratory methods. With the use of mainly in-house developed methods that perform well[23], the standardising of methods was not further explored. The rationale was that standardising methods is important and is encouraged by sharing protocols, but more important is the ability of the laboratory test to

correctly identify RSV. Furthermore, limited progress was made for recommendation 5 on the development of a sentinel approach of hospitals. This recommendation was ranked as a lower priority because non-sentinel data from hospitals are currently being collected. The non-sentinel data could be used for the future establishment of a sentinel laboratory monitoring system and would then need to be assessed for representativeness and quality of data collection.

In this paper we presented data on sentinel RSV and influenza detections. Relatively low numbers of positive RSV tests were reported and this is therefore a limitation. In addition to sentinel data, RSV reports from non-sentinel sources, mainly derived from hospitalised infants are also available and these can provide insight into the epidemic peak of RSV during wintertime. We think that both sources of data are important and complement each other. Sentinel data highlights the occurrence of RSV in the community, where it is an important confounder in influenza surveillance. And hospital-based data present the circulation of RSV in more severe cases and high-risk groups.

The limitations of the sentinel influenza surveillance carried out by EISS are related to differences in case definitions[19], sampling guidelines and laboratory techniques among the different countries.[20] Some difficulty in obtaining swabs from all age groups has been reported, especially for young children in the Netherlands and the elderly in the Netherlands and France.[16] Another limitation is that we could not further investigate other possible causes of respiratory infections such as rhinovirus, adenovirus and coronavirus[26,27] and human metapneumovirus.[28] Country resources however may limit the extension of testing for other viruses in addition to influenza and RSV. Furthermore, no comparison regarding the occurrence of RSV and influenza between the different countries could be made because of differences in data collection procedures and laboratory methods. Additionally, differences in healthcare seeking behaviour may influence the findings between countries.

Currently diagnostic specimens are collected from patients presenting with ILI or ARI. Although ILI and/or ARI case definitions have been used for the detection of influenza for many years, this may not be the optimal clinical indicator for RSV. To investigate the clinical impact and determine the burden of illness of RSV one should extend the diagnostic categories to include acute

bronchitis and otitis media.[29] This may become feasible with the movement towards sentinel networks based on electronic data.
We presented the progress in RSV surveillance based on an influenza surveillance network and data collected for six countries. This illustrated the feasibility of reporting RSV data and showed that a proportion of about 4-18% of the patients were infected with RSV. Sentinel monitoring of RSV and influenza virus is important and may even be extended to other respiratory viruses as the development of multiplex PCR[30] facilitates the detection of other causative agents of respiratory illness. All countries are encouraged to test their specimens for RSV and improvements can be made as less than half of the countries participating in EISS had reported these data. Furthermore, swabbing procedures should be further harmonised and regular quality control of laboratory methods should be performed. When these criteria are met, surveillance of RSV and influenza virus will contribute to a better insight into the burden of respiratory diseases and may be used by healthcare organisations to decide on the timing of palivizumab prophylaxis for RSV in Europe. Overall, this paper illustrated that an existing influenza surveillance system can be relatively easily broadened to include the surveillance of RSV and may be extended to other viruses in the future.

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5

Variation of respiratory syncytial virus and the relation with meteorological factors in different winter seasons

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Abstract

Respiratory syncytial virus (RSV) is the most important viral agent causing severe respiratory disease in infants and children. In temperate climates, RSV activity typically peaks during winter. We have described the seasonal variation in RSV activity and investigated which meteorological variables are related to RSV outbreaks for different time lags. Eleven laboratories in the Netherlands collected data on RSV during the period 1998-2005. Meteorological data were obtained from the Royal Netherlands Meteorological Institute. General linear methods were used to determine the relative contribution of meteorological conditions to reported RSV cases in the winter period. Time lags up to 4 weeks were included to assess a possible delayed weather effect in relation to RSV activity. Onset of RSV activity occurred around week 44 and activity peaked around week 52. Timing of peak activity was very consistent over the study period. Relative humidity was positively associated with RSV activity for all time lags, indicating more RSV when relative humidity increased. Minimum temperature was negatively associated with RSV activity and cloud cover was positively related with RSV activity. Interaction ($p<0.06$) between minimum temperature and relative humidity was observed for a lag of 0, 1 and 2 weeks, indicating that the combination of low temperature and high humidity contributes more to RSV activity than temperature and humidity alone. Relative humidity, minimum temperature, and cloud cover are important predictors of RSV activity in the Netherlands, with the effect of relative humidity being most consistent.

Introduction

Respiratory syncytial virus (RSV) is the most important viral agent causing severe respiratory disease in infants and children worldwide.[1] Most children have been infected at least once by the age of two and re-infections occur throughout life.[2] The symptoms of an RSV infection range from a very mild illness to serious lower respiratory tract infections including bronchiolitis and pneumonia. Although infrequently recognized, RSV is common in adults and can be the cause of severe illness especially in the elderly.[3] The average annual rate of RSV-associated hospitalization has been reported to be 3 (range 2-6) per 1000 children and 17 (range 12-34) per 1000 children under the age of six months.[4] Mortality rates in children are low, RSV attributed deaths have been estimated at 8.4 per 100 000 population. More than half of the attributed deaths in children aged 1 month to 14 years occurred in babies aged 1-12 months.[5]

In temperate climates, RSV activity typically peaks during the winter. Epidemics are related to geographic and climatic factors, but it is not clear whether this is related to the spread of virus, indoor crowding or immunological susceptibility of the population.[6] For the influenza virus, another respiratory virus that circulates during wintertime, transmission is dependent on relative humidity and temperature.[7] This evidence supports the role of weather conditions in the dynamics of influenza and may also be applicable for RSV transmission. However the mode of transmission differs slightly between the two viruses. RSV is transmitted by large-particle aerosols and by direct contact with RSV in solutions of human secretions[8], while influenza is spread via small droplets as people sneeze, cough or talk.

Meteorological conditions such as temperature, relative humidity and UV-B radiation have recently been described in relation to RSV epidemics.[6,9,10] A study performed in Spain indicated that low levels of temperature and low absolute humidity were positively associated with the number of RSV cases and low absolute humidity was independently related to RSV infection.[9] Worldwide, RSV peaks at two temperature intervals: between 2-6°C in temperate regions and 24-30°C in tropical regions. RSV activity was greatest at 45-65% relative humidity and UV-B radiance was inversely related to the number of RSV cases.[10] So far, mainly crude associations between RSV activity and weather have been assessed and correlations between the meteorological variables

have not always been taken into account. In addition, weather models have usually not included a time delay effect on RSV, while one would expect some delay between weather changes and RSV activity.

RSV epidemics occur yearly but may alternate in occurrence between mid-winter and early spring.[11,12] A biannual rhythm with an early RSV season followed by a late season has been described for Finland, Germany and Croatia.[12-14] Year-to-year national and regional variability in the RSV season onset and offset occurs in the United States.[15] Appropriately timed diagnostic testing can provide data that indicate when the RSV season begins nationally and regionally, information that is critical in determining when to begin RSV prophylaxis for infants at high risk for infection. The monoclonal antibody palivizumab offers protection against complications, and the first of the five monthly doses should be administered before onset of community RSV activity. In this paper, we investigate which meteorological factors can predict RSV outbreaks and describe the year to year variation of RSV activity in the Netherlands. To adjust for the incubation time and possible delayed weather effects, four time lags were included in the model to assess the relation of weather variables with RSV activity.

Methods

Design

The role of meteorological variables on RSV was assessed during RSV active periods in eight consecutive years (1998-2005). Data about RSV activity were derived from a central computer-based data system, the Infectious diseases Surveillance Information System (ISIS), based at the National Institute for Public Health and the Environment. Meteorological data were available on a daily basis for ten variables and collected by the Royal Netherlands Meteorological Institute (KNMI). We used the number of RSV confirmed cases per week as the outcome variable and investigated whether there was an association between weather variables and RSV in the Netherlands.

Selection of RSV active periods

All analyses were restricted to RSV active periods, starting at week 40 and ending in week 20 of the following year. The season onset and offset criteria for RSV were based on those of the National Respiratory and Enteric Virus

Surveillance System.[16] We defined the week of onset as the first of two consecutive weeks in which at least 10% of the tested samples tested positive for RSV and a minimum of 10 samples were tested in that week. Offset was defined as the last of two consecutive weeks with at least 10% of the samples tested positive for RSV and a minimum of 10 samples tested in that week. All seasons included a week 53 that usually comprised one or two days. For defining the onset and offset, we have compared the threshold of 10 tests per week to a threshold of 20 tests per week. The threshold of 20 samples showed little difference for the onset, but large difference in defining the offset. We defined the threshold at 10 tests per week, which was most sensitive in determining the onset and offset of the RSV active period. The duration was defined as the number of weeks from onset to offset.

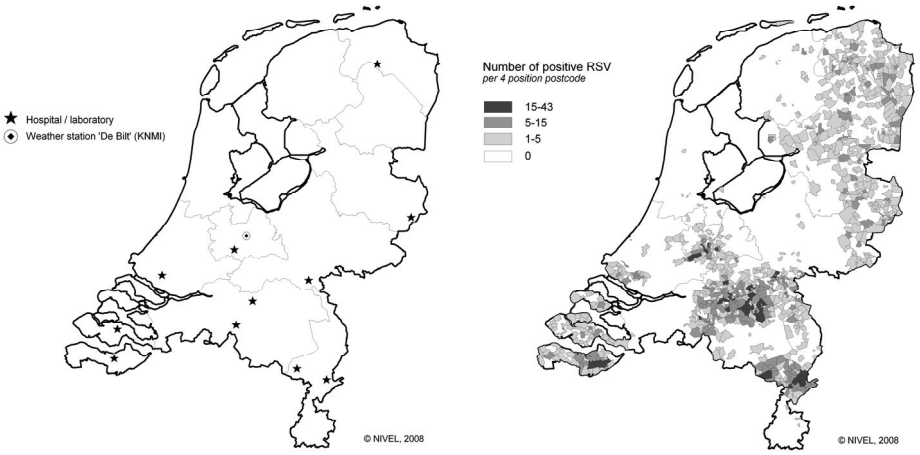
RSV data

Data were derived from ISIS. This system automatically collects data on a daily basis on RSV from regional laboratories and/or hospitals in the Netherlands and these were mainly concentrated in the middle and southern regions of the Netherlands (Figure 5.1). Data from eleven laboratories were included and contained information on the age of the patient, gender, four digit postal code, type of material sampled and the laboratory method. Both positive and negative test results were recorded. All test results were anonymously and uniquely coded for each patient.

To estimate the population coverage of the laboratories, we looked at the laboratories that reported to ISIS in 2000 (n=9) and compared this to the total number of laboratories (n=58) in the Netherlands as a whole.[17] The coverage of the ISIS laboratories was estimated at 2.4 million, which covers around 16% of the Dutch population. It was assumed that all patients with severe RSV infection in the region would be picked up by the regional laboratory. Participating laboratories generally used one or two diagnostic methods: (in) direct immuno-fluorescence (n=6), culture (n=5), antigen detection (n=3), or enzyme-linked immunosorbent assay (ELISA) (n=2). In the Netherlands, an attempt is usually made to come to an RSV diagnosis in hospitalized bronchiolitic infants. Diagnostic tests are rarely performed in non-hospitalized children with respiratory symptoms.

Figure 5.1: Geographical distribution of the laboratories that reported RSV data (a), and the number of RSV cases in the different postal codes reported by the laboratories in the period 1998-2005 (b)

a. b.



Meteorological data

The Royal Netherlands Meteorological Institute (KNMI) collects daily meteorological data in the Netherlands. Data was obtained from a centrally located weather station (De Bilt) and included: prevailing wind direction in degrees (360=North, 180=South, 270=West, 0=calm/variable), daily mean wind speed in 0.1 m/s, daily mean temperature in 0.1 degrees Celsius, minimum temperature in 0.1 degrees Celsius, maximum temperature in 0.1 degrees Celsius, sunshine duration in 0.1 hour, percentage of maximum possible sunshine duration, daily precipitation amount in 0.1 mm (-1 for < 0.05 mm), daily mean surface air pressure in 0.1 hPa, cloud cover in octants (0 = no clouds – 9 = sky invisible), and daily mean relative atmospheric humidity in percentage.

The absolute humidity is directly related to the temperature and relative humidity, and was calculated by multiplying relative humidity with water vapor, as has been previously described in Lapena *et al*.[9] For the prevailing wind direction, we categorized the four wind directions and calculated the most frequently observed wind direction in the form of the modus per week.

The weekly sum of precipitation data was used, and for all other weather variables the data were aggregated to the week average value.

Data analysis

The outcome variable was the number of positive cases of RSV per week during RSV active periods. Multiple linear regression analysis was performed on all weekly aggregated weather variables and the number of cases of RSV for all years. Variables were checked for correlation. Poisson and negative binomial regression analyses were performed in addition to the linear regression analysis. Week 53 consisted of one or two days and the RSV cases for this week were extrapolated to a whole week for the analysis. Weather data for week 53 were not extrapolated, because all weather variables, except precipitation, were aggregated to the week average value.

Because the incubation time of RSV infection is 2-7 days and there might be a delay in the weather effect in relation to RSV infection, different time lags (no lag, lag of one week, lag of two weeks, lag of three weeks, lag of four weeks) were included in the regression analyses. The descriptive and linear regression analyses were performed using SPSS 14. STATA 9 was used for the Poisson and negative binomial regression analysis and the robust option was used to adjust for heterogeneity in the model.[18] Significance was concluded when $p<0.05$.

The interaction between relative humidity and minimum temperature was calculated by multiplying the two variables. The minimum temperature was multiplied by -1 before calculating the interaction because this variable was negatively associated with RSV activity and opposite to the association of RSV with relative humidity. Significance of interaction was concluded when $p<0.1$.

Results

Demographics

Data from eleven laboratories for the period 1998 to 2005 were included in the analyses. A total of 10672 tests were performed during the RSV active periods. Baseline characteristics of the participating laboratories can be found in Table 5.1. Most cases were infants aged below 6 months and more boys than girls tested positive for RSV which corresponds with previous studies.[19,20]

Table 5.1: Baseline laboratory/hospital and patient characteristics in the period 1998-2005

Laboratory/hospital	Period reporting in winter seasons	Number of tests	Number of positive RSV tests (%)	Median age in months (range)	Gender (% male)
1) Bosch Medisch Centrum, Den Bosch	98/99-04/05	2235	1090 (49%)	4 (0-149)	57
2) Canisius Wilhelmina Hospital, Nijmegen	99/00-02/03	853	274 (32%)	4 (0-44)	54
3) Laboratory for Infectious Diseases, Groningen	02/03-05/06	2669	751 (28%)	4 (0-975)	61
4) Laboratorium Microbiologie Twente Achterhoek, Enschede	00/01,02/03 -05/06	669	233 (35%)	5 (0-86)	64
5) Ruwaard van Putten Hospital, Spijkenisse	01/02-05/06	341	123 (36%)	4 (0-39)	57
6) St. Antonius Hospital, Nieuwegein	98/99-05/06	1001	393 (39%)	4 (0-965)	59
7) St. Elisabeth Hospital, Tilburg	02/03-05/06	720	253 (35%)	3 (0-43)	55
8) St. Jans Gasthuis, Weert	98/99-05/06	415	160 (39%)	4 (0-40)	60
9) St. Laurentius Hospital, Roermond	98/99-05/06	890	416 (47%)	5 (0-85)	55
10) Streeklaboratorium Zeeland, Terneuzen	98/99-05/06	686	208 (30%)	5 (0-39)	57
11) Streeklaboratorium Zeeland, Goes	99/00-05/06	193	101 (52%)	3 (0-52)	65
Total		10672	4002 (38%)		

Seasonal trends

The number of participating laboratories increased from five in 1998 to eleven in 2002-2003 (Table 5.2). In 2005, the season only contained data up to week 52, and therefore fewer cases were reported for this season. The onset of RSV activity occurred around week 44, peaked around week 52, and was quite consistent for the study period. The duration and offset differed somewhat by season. Relative humidity and cloud cover were similar for the different seasons around both the onset and the peak of RSV activity. Some differences in the minimum temperature are observed for the different winters with a generally lower temperature around the peak (Table 5.2).

Table 5.2: Overview seasonality RSV activity and meteorological variables by winter season for the period 1998-2005

Year	RSV cases**	Number labs	Week onset	Week peak	Week offset	Week duration	Minimum temp. *(degrees Celsius)* onset	Minimum temp. *(degrees Celsius)* peak	Relative humidity (%) onset	Relative humidity (%) peak	Cloud cover in octants onset	Cloud cover in octants peak
1998-99	95	5	50	51	2	5	5.4	2.2	95	91	7	6
1999-00	462	7	44	52	12	22	6.1	1.8	87	90	5	6
2000-01	452	8	45	50	15	24	5.8	7.8	89	86	7	7
2001-02	410	8	43	51	11	22	8.6	-2.8	91	91	6	5
2002-03	439	11	42	50	13	25	5.2	-4.1	90	80	6	6
2003-04	811	10	47	51	17	27	7.6	-0.2	92	89	7	5
2004-05	917	10	45	52	11	20	4.5	0.3	88	90	6	5
2005-06*	353	9	44	52	n.a.	n.a.	10.7	-2.1	82	89	5	6
Median	446	9	45	51	12	22	6.0	0.1	90	90	6	6
Mean	492	9	45	51	12	21	6.7	0.4	89	88	6	6

* Data available till week 52/2005; ** original data, no correction for week 53; n.a.: not applicable due to limited data.
Onset : first of two consecutive weeks with at least 10% positive for RSV and at least 10 samples tested in that week.
Offset : last of two consecutive weeks with at least 10% positive for RSV and at least 10 samples tested in that week.
Duration : number of weeks from onset to offset including week 53 (usually one or two days).

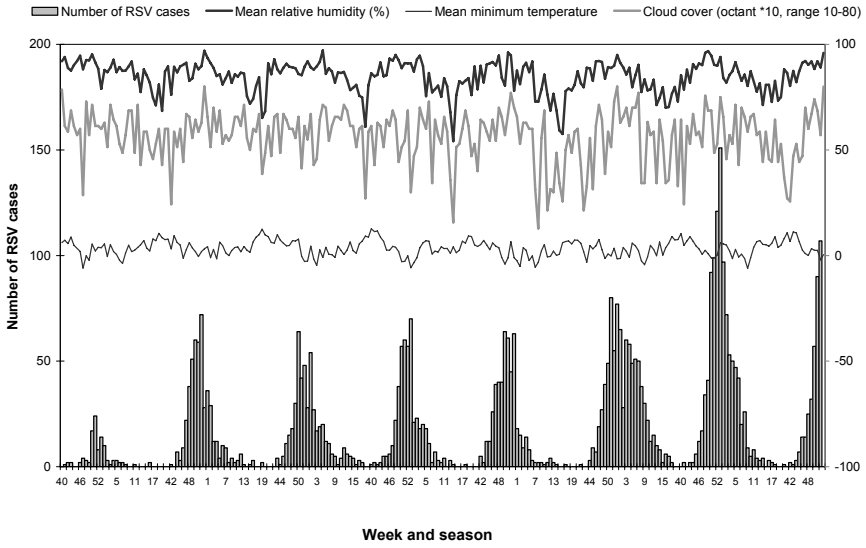
Association with meteorological factors

The consistent onset would suggest some factor that triggers the RSV activity. This factor may be related to the weather. We assessed whether the weather variables were correlated with the weekly number of RSV cases. The first step was to assess the correlation between weather variables. A strong correlation ($r > 0.8$) was observed for 1) minimum temperature and absolute humidity, 2) relative humidity and sunshine duration, and 3) cloud cover and sunshine duration. Additionally, correlation was observed for the three temperature variables: minimum, mean and maximum temperature. We therefore excluded the following variables from the analyses: absolute humidity, sunshine duration, mean temperature and maximum temperature. The minimum temperature and relative humidity have been suggested as predictors of RSV activity in earlier studies[9,10] and were included in the analysis, as well as the other weather variables that did not present a high correlation with other weather variables.

The wind speed, modal prevailing wind direction, minimum temperature, precipitation, air pressure, cloud cover and relative humidity were assessed on the relation with weekly number of RSV cases (Pearson correlation). The relative humidity ($r = 0.340$), minimum temperature ($r = -0.338$), and cloud cover ($r = 0.221$) were significantly correlated with the weekly number of RSV cases and this finding was observed for all time lags, although the r-value decreased with increasing time lag for relative humidity and cloud cover.

We also emphasized to illustrate the relation of weather variables with RSV activity. The weekly number of RSV positive patients is presented for the weeks 40 to 20 during 1998-2005, as well as the weather variables minimum temperature, relative humidity and cloud cover (Figure 5.2). For the three weather variables the weekly values were quite variable. The temperature at the beginning and end of the season was higher compared to the middle of the season when RSV activity was at its peak. A general drop in temperature around the RSV peak was most obvious for the period 2000-2003. Relative humidity was generally higher around the RSV peak, while at the end of the season a sudden drop in relative humidity can be observed. The figure presenting cloud cover and RSV also shows that RSV peaked around the time the highest cloud cover was reported. Although the weekly variation of the variables makes it difficult to track any subtle effects, this figure allow us to visualize a relation of meteorological factors with RSV.

Figure 5.2: RSV detections and minimum temperature (°C), relative humidity (%) and cloud cover (octant*10) in the winter periods 1998-2005 in the Netherlands. The weekly mean values of the weather variables are presented on the y-axis



Multiple linear regression analysis was initially performed for each of the years separately (data not shown). At least one of the meteorological factors, minimum temperature, relative humidity and cloud cover, was significantly associated with RSV activity in seven out of eight winters seasons: 1998-99, 2000-01 to 2004-05. Relative humidity was a significant factor in the winter of 2000-01, 2001-02, 2003-04, 2004-05 for multiple time lags. Temperature was significantly associated with RSV in four winters, varying from lag = 0 to lag = 2. Cloud cover was significantly associated with RSV in three winters for lag = 0 and/or lag =1.

For all seasons together, we performed multiple linear regression analysis and evaluated the model with the number of RSV cases as dependent variable, and the relative humidity, minimum temperature and cloud cover as independent variables. This model explained about 21% of the variance in RSV cases (R^2 = 0.209; see Table 5.3). When looking at the same model for different time lags,

which ranged from one to four weeks, the effect of the weather variables decreased: R^2 from 0.209 to 0.165.

The relation of temperature with RSV was negative, indicating more RSV activity when the temperature decreased. A positive relation was observed for relative humidity and to some extent for cloud cover, indicating more RSV when the relative humidity or cloud cover increased. The minimum temperature contributed to the RSV cases for most time lags (0-3 weeks). The relative humidity still played an important role at the time lag of 4 weeks (Table 5.3). Cloud cover was borderline significant (p=0.051) in relation with RSV in the first time lag. In order to investigate whether the effect of one meteorological variable on RSV activity depends on the level of a second weather variable we calculated the interaction effect of minimum temperature and relative humidity on RSV activity and included this in the regression model (Table 5.4). There was an interaction effect at lag = 0 (p=0.018), lag = 1 (p=0.053) and lag = 2 (p=0.059) and the role of temperature was reduced and borderline significant at lag =1.

Table 5.3: Multiple linear regression analysis with the number of RSV cases as the dependent variable, and minimum temperature, relative humidity and cloud cover as independent variables for different time lags expressed in standardized beta coefficients and explained variance (R^2)

Weekly number RSV cases	Weekly mean min. temperature	Weekly mean relative humidity	Weekly mean cloud cover	R^2
No lag	-0.321**	0.203**	0.141[a]	0.209
Lag 1 week	-0.243**	0.282**	0.123	0.201
Lag 2 weeks	-0.226**	0.358**	0.046	0.206
Lag 3 weeks	-0.153*	0.404**	-0.014	0.185
Lag 4 weeks	-0.043	0.433**	-0.071	0.165

* p< 0.05 ** p< 0.01.
[a] The p-value is borderline significant: p = 0.051.

Table 5.4: Multiple linear regression analysis with the number of RSV cases as the dependent variable, interaction between minimum temperature and relative humidity, minimum temperature, relative humidity and cloud cover as independent variables for different time lags expressed in standardized beta coefficients and explained variance (R^2)

Weekly number RSV cases	Interaction minimum temp and relative humidity[a]	Weekly mean min. temperature	Weekly mean relative humidity	Weekly mean cloud cover	R^2
No lag	1.566*	1.256 [b]	0.339**	0.142*	0.227
Lag 1 week	1.359[b]*	1.120	0.389**	0.124	0.213
Lag 2 weeks	1.581[c]*	1.354	0.464**	0.043	0.219
Lag 3 weeks	1.339	1.183	0.487**	-0.014	0.193
Lag 4 weeks	1.044	0.996	0.491**	-0.071	0.166

Note: Interaction between minimum temperature and relative humidity calculated by (minimum temperature (*-1)) * (relative humidity).

For the three weather variables:
* $p < 0.05$
** $p < 0.01$
[a] For the interaction term significance was concluded when $p<0.1$.
[b] $p = 0.053$.
[c] $p = 0.059$.

In addition to multiple linear regression analysis, Poisson regression was carried out as count data are highly non-normally distributed and are better estimated by Poisson regression. The results of the Poisson regression analysis were identical for the minimum temperature and relative humidity as performed by the linear analysis (data not shown). In the Poisson regression cloud cover was a significant factor at no lag ($p<0.01$) and a lag of one week ($p<0.05$) where the linear regression found a borderline significant effect at no lag ($p = 0.051$). Poisson regression assumes that the mean and the variance are the same; our dataset did not fit this assumption. Therefore we also performed negative binomial regression, which is better fit for over-dispersed data. For all three meteorological factors the previous Poisson analysis was confirmed.

Discussion

Our data showed that relative humidity, minimum temperature and cloud cover were important predictors of RSV activity for different time lags in the Netherlands. The effect of the time lag is relevant and the three meteorological factors contributed most to the model at lags 0 to 2 weeks, indicated by the R^2 explaining the variance in RSV cases for about 22%. The effect of meteorological factors was not statistically significant for all individual winter seasons, but this was expected due to the loss of power in particular the 1998-99 and 1999-00 winters when a low number of samples were tested for RSV in these periods.

Most obvious was the positive relation of RSV with the relative humidity that showed significant association for all time lags, also after adding an interaction term into the regression model. Minimum temperature was inversely related to RSV and this parameter contributed to RSV activity in three out of the four time lags. After addition of the interaction parameter into the model the effect of minimum temperature decreased. The interaction found between relative humidity and minimum temperature highlighted that the combination of the two parameters have a stronger impact on RSV activity than each of the parameters alone and better fitted the model as R^2 was higher.

The consistent peak week of RSV activity observed for the Netherlands has also been reported for the United Kingdom, but differs from reports made for Croatia and Finland. In Croatia and Finland RSV outbreaks occur in every second year causing a mild spring peak, followed by a larger outbreak in the winter during the same year. For Croatia the large RSV outbreak was inversely related to the temperature and directly to humidity[13]. But the subsequent winter is rather silent, and the role of meteorological factors did not explain the timing of RSV outbreak in Croatia. Differences in outbreaks between countries may be related to the climate and population density. The Netherlands has a temperate, marine climate, similar to the United Kingdom. Croatia has a Mediterranean and continental climate, while Finland has a cold temperate climate. Population density in the Netherlands is 395 inhabitants/km^2, while in Finland it is 16 inhabitants/km^2 and in Croatia 81 inhabitants/km^2.[21] The silent winter might be explained by the effect of the low population and thereby a low number of susceptible children, which lowers spread of RSV.

The relation between RSV activity and low minimum temperature may indicate that a low temperature enhances virus transmission. It has been described in earlier studies that transmission of RSV is inversely related to temperature in cooler climates and this may be a result of increased stability of the virus in the secretions in the colder environment.[10] A limited amount of literature is available on RSV transmission and the role of temperature and humidity, but has been investigated for influenza, also a typical winter disease. Lowen et al. (2007) found that influenza virus transmission is dependent on relative humidity and temperature and provided direct, experimental evidence to support the role of weather conditions in the dynamics of influenza. They found increased shedding of virus and enhanced transmission at 5°C when compared to 20°C. Research on the stability of RSV has been described by Rechsteiner and Winkler[22], they prepared stable aerosols of RSV and kept them at different relative humidity. Virus recoveries were highest at high relative humidity and the stability of the aerosol was maximal at 60% relative humidity. This indicates that humidity indeed plays an important role and may affect transmission of the virus.

Our study also highlighted a probable effect of cloud cover on RSV. To our knowledge, no other study has included this meteorological variable in the analysis. However, UV B has also been reported to predict respiratory syncytial virus activity.[10] A possible explanation is that UV B radiance could interfere with the spread of RSV by inactivating the virus. UV B could also indirectly affect RSV activity by stimulating vitamin D on the outcome of RSV infection. A number of studies have indicated that vitamin D deficient children are at greater risk of having respiratory infections.[23,24] In our study we did not include UV B, however one might expect some relation between UV B and cloud cover. Moise and Aynsley indicated that increasing cloud cover decreases the UV B because of the increase in the diffuse fraction of the radiation, and for the Netherlands it has been estimated that clouds generally decreased the UV radiation by 34% in the period 1998-2005.[25,26] The direction of the relation of cloud cover corresponds to the inverse relationship of RSV activity and UV B that has been described by Yusuf et al.[10]

One limitation of this study was the number of laboratories that reported data to ISIS; the number varied by year and thereby affected the number of tests performed for RSV diagnosis. In addition the methods used differed somewhat by laboratory, and did not include the most sensitive methods such as real-

time polymerase chain reaction. Another limitation concerns the analysis of meteorological data as we chose to calculate weekly averages for most factors and modus for the wind direction. For some variables the values can differ considerably by day, e.g. wind direction, and our procedure may have reduced sensitivity to pick up the daily variation, and smoothing the effect of the weather variables on RSV. Finally, we used meteorological data from a central point, "the Bilt", in the Netherlands, while using RSV data from predominantly the Southern and Eastern provinces of the country. However, we do not think that regional differences in weather would have affected our main outcomes, as distances are quite small in the Netherlands. In addition, although some minor daily variations in meteorological factors exist, we do not think that the slight differences in temperature and humidity have an effect on the study outcome, also because data was averaged to a weekly value.

Factors other than meteorological conditions contribute to the spread of RSV. Meteorological factors in our study explained part (21%) of the variation in RSV cases; a driving factor for RSV activity is the immunity against RSV among the susceptible young population. Furthermore, weather factors might also have their effect through the behavior of the population when RSV is circulating. One practical explanation could be that in cold, rainy and cloudy days people stay inside and this causes more indoor crowding and transmission. In addition cold weather may also have an effect on the respiratory tract, as cold air may induce broncho-constriction.[27,28]

The onset of RSV activity was very consistent for the eight winters and was around week 44, which is similar as described for the UK where the incidence of acute bronchitis and bronchiolitis in young children revealed a consistent increase in RSV activity during week 43 each year.[29] Practical implications of our study relate to timing of RSV prophylaxis. The monoclonal antibody palivizumab offers protection against complications, and the first of the five monthly doses should be administered before onset of community RSV activity. Our findings of a consistent increase in RSV around week 44 in the Netherlands could be used for RSV prophylaxis, preferably combined with real-time RSV surveillance.

The theoretical implications of our study are that relative humidity, minimum temperature and cloud cover are important predictors of RSV in the Netherlands and may be related to transmission of the virus. More

experimental research however is needed. In summary, we have found that onset of RSV activity is quite consistent in the Netherlands and that relative humidity, minimum temperature and cloud cover predict RSV activity in the Netherlands, with the effect of relative humidity being most consistent.

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6

The impact of laboratory characteristics on molecular detection of respiratory syncytial virus in a European multicentre quality control study

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Abstract

The laboratory performance of nucleic acid amplification techniques for respiratory syncytial virus (RSV) diagnosis was investigated in 25 laboratories across Europe. Our aim was to assess the laboratory performance of nucleic amplification tests and to explore what factors were related to the diagnostic performance. The panel consisted of nine samples containing RSV-A and/or RSV-B and one negative sample. Qualitative results and data on the type of assay and other laboratory characteristics were collected. An explorative analysis was carried out to investigate whether the laboratory characteristics were related to the diagnostic performance of the laboratories. The overall sensitivity for all laboratories was 88% (n=25; range 50-100). A correct score of 93% (range 70-100) was observed for laboratories performing in-house real-time PCR or nested PCR. Multilevel analysis showed that the type of assay (nested or real-time PCR vs. commercial test) was a significant factor (OR=8.39; $CI_{95\%}$ 1.91-36.78) in predicting a correct result. The results for this external quality control study show that the overall performance of laboratories for RSV diagnosis in Europe is good and that real-time PCR is preferably used for RSV diagnostics.

Introduction

Respiratory syncytial virus (RSV) is a major cause of lower respiratory tract infections, particularly in infants and the elderly[1-3] and leads to hospitalizations and excess mortality.[4] Diagnostic RSV testing is usually performed on infants, elderly patients, and those with compromised immune systems, who have moderate to severe symptoms and lower respiratory tract involvement. RSV testing is also carried out to define and track the spread of RSV in the community.[5]

Treatment of RSV infection is primarily supportive (minimizing pain and fever and easing breathing), however palivizumab, a monoclonal antibody, is available for RSV prophylaxis in preterm and high risk infants. Although antibiotic use is not indicated for bronchiolitis[6] caused by RSV, a substantial proportion of RSV infected children (45%) received antibiotics.[7] This inappropriate treatment can possibly be avoided by early laboratory diagnosis

of RSV infection.[8] A rapid, valid and sensitive method for diagnosis is therefore important.

One way to validate diagnostic tests is to participate in external quality assessment studies. External quality control for molecular detection has been reported for different pathogens including respiratory viruses.[9-11] This type of assessment of laboratory diagnostics is important because assays continue to evolve and external quality control studies can contribute to further improvement of diagnostic methods. Rapid diagnostic RSV tests with a high sensitivity may result in improved patient care by decreasing length of hospitalization and directing specific therapy.[12]

Over the last decade, nucleic amplification techniques (NATs) have become the new standard for virus detection and have moved into the mainstream of clinical testing.[12] The major advantages of NATs compared to conventional techniques are the high sensitivity and speed.[13,14] However, many NATs are still technically demanding and susceptible to contamination. Few factors[11,15] have been described to help improve laboratory diagnostics and are related to the prevention of contamination and the choice of gene target.

The European Influenza Surveillance Scheme (EISS) and the organization Quality Control for Molecular Diagnostics (QCMD) carried out a molecular external quality control study for RSV and compared the laboratory performance of NATs for RSV diagnosis of different reference laboratories in western and eastern Europe. This is the first study that explores whether laboratory characteristics are related to the quality of the diagnostic methods. The overall aim was to assess the laboratory performance of RSV molecular assays and to provide insights into laboratory specific factors that may be used to improve the quality of RSV molecular diagnostic methods.

Materials and Methods

Panel composition and participants

The panel was distributed by the QCMD Neutral Office in Glasgow, United Kingdom. QCMD is specialized in the provision of External Quality Control Assessment schemes for a wide range of pathogens. The QCMD RSV panel consisted of nine coded samples containing RSV-A and/or RSV-B (ATCC strain

RSV-A-2; ATCC strain RSV-B/WV/14617/1985 wild type), and one sample negative for RSV (Table 6.1). The matrix was Dulbecco's Modified Eagle's Medium and 10% Foetal Calf Serum. No quantitative data were available for the RSV panel, only sample dilutions; this procedure allows sample uniformity and reproducibility.

Independent testing was performed by three laboratories in May 2006 and ensured the quality of the samples. The panels were distributed on dry ice by courier service to 33 laboratories (23 countries) on 27 June 2006. To ensure confidentiality, all participating laboratories received a code number. The selected participating laboratories were generally the national reference laboratories for influenza who take part in EISS.[16,17]

Data collection

Participants returned results to QCMD and completed a technical online questionnaire within six weeks after distribution of the panel. Information on the type of assay (commercial test, single PCR, nested PCR, real-time PCR), number of RSV tests performed per year (<500; ≥500), and the accreditation of the laboratory were collected through the QCMD technical questionnaire. Additionally, a questionnaire was sent electronically to the participating laboratories. This questionnaire included information on the following items: date of receipt of the panel (no delay, ≥ one week delay), training level of the laboratory staff (no training: "no training or doctoral studies", training: "general training and experienced personnel", PCR training: "specific PCR training"), target gene, whether the sample was tested for inhibition of the amplification reaction, and whether the participating laboratory was the national reference laboratory for RSV. It was hypothesized that these factors could be determinants of the performance score, and were therefore included in an explorative analysis to see whether these items were related to the performance of the laboratories.

To investigate whether participants from different European regions achieved similar results, countries were divided into eastern and western European countries. On the basis of the United Nation Statistics Division the following countries were defined as "east": the Czech Republic, Lithuania, Latvia, Poland, Romania, Slovenia, Slovakia, Estonia. Western European countries were: Austria, Denmark, Finland, France, Germany, Great Britain, Greece,

Ireland, Italy, Malta, Netherlands, Norway, Portugal, Spain, Sweden and Switzerland.

Performance score

A sum score was defined and used to analyze the performance of the laboratories. For each correct result on a positive or negative sample one point was given; no points were given in case of an incorrect result. The maximum score of the laboratory was 10 points. For data analysis the percentage of correct results was calculated. Typing of RSV was not performed by all laboratories and therefore typing results were not taken into account in calculating the performance score. Four respondents returned two datasets from two testing procedures of the same panel. We included the first submitted dataset in the analysis. Results for the first and second dataset were identical for three out of four respondents, one laboratory had a lower score for the second dataset (7 vs 10 points). The analyses were performed by the first author who was blinded for the laboratory code.

Statistical analyses

Statistical significance of differences in the mean performance score for different subgroups of variables was calculated using the Mann-Whitney U Test. This non-parametric test was used because the results for the performance score were skewed to the right and sample size was small. The Kruskall-Wallis Test was conducted to compare the performance scores for type of assay and training level. Significance was concluded when $p<0.05$. The program SPSS 14.0 was used for the analyses.

Multilevel logistic regression was used to determine which laboratory characteristics were the best predictors of a correct result on each of the ten samples. We chose a multilevel analysis as the performance score for one sample is not independent of the performance score of another sample of the same laboratory; multilevel analysis takes into account such a hierarchical structure. Laboratories with missing data on the question of training level and the number of swabs tested in a year (n=7) were excluded from the multilevel analysis.

Multilevel logistic regression analyses with a random intercept were performed using the second order PQL method.[18] For the dependent variable (sample score: incorrect = 0; correct = 1) a logistic regression model was calculated

with the following laboratory characteristics as independent variables: type of assay (two dummy variables ("real-time PCR or nested PCR" and "single PCR"; reference category "commercial assay"), level of training (two dummy variables; reference category "no training") and number of swabs tested (<500 or ≥500). We combined the real-time and nested PCR in the multilevel analysis as they showed similar performance scores in the quality control assessment. All multilevel analyses were performed with MLwiN[19], a statistical program for multilevel analyses.

Results

Participants

The panels were distributed to 33 laboratories (23 countries). Eight participants did not return results for this program of which three withdrew officially. Reasons for the three laboratories to withdraw were: assay not available, resource issues and inconclusive results. The number of participating laboratories was 25 (76%) from 18 countries. Of the 25 participating laboratories 20 laboratories were from western Europe and five were from eastern Europe.

Performance score

The percentage of correct results on sample level ranged from 60% for sample 9 to 100% for samples 1, 2, 6 and 7 (Table 6.1). The percentage correct results decreased correspondingly with decreasing sample concentration. One false positive test result (4%) was reported for the negative panel sample. The rate of false negatives was 14%. Lowest correct performance scores on sample level were observed for samples containing RSV-B only: 60% in sample 9, and 72% in sample 5. Laboratories that did not detect RSV-B in sample 9 were not able to detect RSV-B in sample 5 either. Two samples contained both RSV-A and RSV-B (samples 2 and 6), with RSV-B at a dilution of $2.0x10^{-4}$ for both samples (Table 6.1). We compared the laboratories that reported RSV-B in these samples (n=14) to laboratories that did not detect RSV-B in the more diluted samples 5 and 9 ($1.0x10^{-5}$). Two laboratories detecting RSV-B in specimens 2 and 6 were not able to detect RSV-B in specimens 5 and 9, indicating a problem in the sensitivity of the assay performed in these laboratories.

Table 6.1: Panel composition and percentage correct results

Sample code	Sample content	Target sample dilution	Number correct results	Percentage correct results (N=25)
1	RSV A	1.0x10-5	25	100%
2	RSV A and B	2.0x10-5 / 2.0x10-4	25	100%
3	RSV A	1.0x10-6	19	76%
4	RSV Negative		24	96%
5	RSV B	2.0x10-5	18	72%
6	RSV A and B	2.0x10-5 / 2.0x10-4	25	100%
7	RSV A	1.0x10-5	25	100%
8	RSV A	2.0x10-6	21	84%
9	RSV B	2.0x10-5	15	60%
10	RSV A	2.0x10-6	22	88%

Performance and type of assay

The performance for the individual laboratories is presented in Table 6.2. Eleven laboratories reported 100% correct results. The overall mean sensitivity was 88% (range 50-100). The majority (22 out of 25) of laboratories used an in-house assay. In particular real-time PCR and nested PCR assays provided the highest performance scores (93% correct score; range 70-100) and were used in 19 laboratories. Three laboratories used commercial assays and the percentage correct results, representing the overall score of the laboratory, ranged from 50% to 80%. Laboratories that had a performance score of 80% or lower usually had difficulty in the detection of RSV-B (Table 6.2).

Table 6.2: Performance score and type of assay per laboratory

Lab code* (N=25)	Score by sample type				Score total	Type of assay	
	RSV A (n=5)	RSV B (n=2)	RSV A and B (n=2)	RSV negative (n=1)	% correct score		
2	5	2	2	1	100	In-house	Realtime PCR
4	5	2	2	1	100	In-house	Realtime PCR
10	5	2	2	1	100	In-house	Realtime PCR
15	5	2	2	1	100	In-house	Realtime PCR
16	5	2	2	1	100	In-house	Realtime PCR
29	5	2	2	1	100	In-house	Realtime PCR
7	5	2	2	1	100	In-house	Nested PCR
12	5	2	2	1	100	In-house	Nested PCR
28	5	2	2	1	100	In-house	Nested PCR
32	5	2	2	1	100	In-house	Nested PCR
6	5	2	2	1	100	In-house	Multiplex Nested-PCR
5	4	2	2	1	90	In-house	Realtime PCR
27	4	2	2	1	90	In-house	Realtime PCR
3	5	1	2	1	90	In-house	Nested PCR
1	4	2	2	1	90	In-house	Semi-Nested PCR
11	5	1	2	1	90	In-house	2 step RT-PCR
20	5	0	2	1	80	In-house	Single PCR
31	4	1	2	1	80	In-house	Realtime PCR
13	5	0	2	1	80	In-house	Nested with RT 2nd Rnd
22	5	0	2	1	80	Commercial	Arrow Diagnostic Fast set RSV A/B
26	2	2	2	1	70	In-house	Realtime PCR
9	4	0	2	1	70	In-house	Nested PCR
21	5	0	2	0	70	In-house	Single PCR
18	3	0	2	1	60	Commercial	Euroclone RSV-check kit
33	2	0	2	1	50	Commercial	Finnzymes RobusT II RT-PCR Kit

The gene target for amplification varied between the laboratories, though most laboratories used the nucleoprotein gene as gene target, singly or in a combination with another gene (Table 6.3). To test whether a combination of target genes showed a better performance than a single target gene, we performed the Mann-Whitney U test. The performance of laboratories that used a combination of target genes (n=4) did not differ significantly (p=0.076) from laboratories that used one target gene (n=19).

Table 6.3: Performance score by target gene

Target gene	(N)	Percentage correct result (%)	
		median	range
N/G	1	100	
N/P	3	100	90-100
N or NS-1*	1	90	
F	5	90	70-100
N	11	90	50-100
L	1	80	
G	1	60	

* N for RSV A; NS-1 for RSV B.
Abbreviations: N, nucleoprotein gene; P, phosphoprotein gene; G, surface glycoprotein gene; L, polymerase gene; F, surface fusion protein; NS-1, non-structural protein 1.

Performance and laboratory characteristics

The laboratory performance score is presented for the different laboratory characteristics in Table 6.4. The Mann-Whitney U Test or Kruskall-Wallis Test was conducted to compare the performance for the different laboratory characteristics. Statistically significant differences in means were observed for the type of assay ("commercial", "single PCR", "nested PCR", and real-time PCR": p=0.026). These results suggest that there is a difference in performance scores across the different types of assay, with the highest performance scores for laboratories using real-time PCR and nested PCR.

No significant differences were observed for the performance score across different level of training (p=0.080) and the number of swabs tested (p=0.086). Furthermore, none of the other laboratory characteristics had an impact on the laboratory performance (Table 6.4).

Table 6.4: Percentage correct result for the different laboratory characteristics

Laboratory characteristics	N	Percentage correct result (%)		
		median	range	p-value
Type of assay				
Commercial	3	60	50-80	0.026
Single PCR	3	80	70-90	
Nested PCR	9	100	70-100	
Real-time PCR	10	100	70-100	
Number of swabs tested				
<500	9	90	50-100	0.086
>500	10	100	70-100	
Laboratory accreditation				
No	7	100	50-100	0.723
Yes	12	90	70-100	
Delay in receipt panel				
No delay	15	100	60-100	0.199
≥ One week delay	10	85	50-100	
Training level				
No training	5	70	50-100	0.080
Internal training	13	90	70-100	
PCR training	5	100	90-100	
Samples tested for inhibition				
No	18	90	50-100	0.944
Yes	6	95	70-100	
National reference laboratory				
No	17	90	50-100	0.539
Yes	5	90	80-100	
Region				
East	5	100	50-100	0.567
West	20	90	60-100	

Multilevel analysis

To study the effect of each of the relevant variables corrected by other variables on the performance score, multilevel logistic regression analysis was performed (Table 6.5). The data was analyzed on sample level, with 10 samples for each laboratory (N=18). We included the following variables in the model: type of assay, level of training and the number of swabs tested in a year in the model. The results showed an OR of 8.39 ($CI_{95\%}$: 1.91-36.78) for the in-house PCR (nested or real-time PCR) versus commercial PCR. The other variables did not predict independently a good performance.

The different types of assay were also assessed separately and compared using contrast test for fixed effects in MLwiN resulting in a chi-square test statistic. A significant difference in performance score between commercial and nested PCR (X^2= 12.92; p<0.001) and between commercial and real-time PCR (X^2 =14.62; p<0.001) were observed. Additionally significant differences were observed between single PCR and nested PCR (X^2 = 3.86; 0.02<p<0.05) and single PCR and real-time PCR (X^2 = 4.82; 0.02<p<0.05).

Table 6.5: Multilevel analysis (N=10 tests; N=18 laboratories): Predictive factors for a correct result on sample level adjusted for the type of assay, level of training and number of swabs tested in a year

	OR	CI (95%)
Type of assay: single PCR*	2.21	0.51 - 9.67
Type of assay: nested or real time PCR*	8.39	1.91 - 36.78
Level of training: internal**	1.15	0.34 - 3.96
Level of training: PCR**	2.31	0.22 - 24.36
Number of swabs tested	0.87	0.25 - 3.00

* Dummy variable, reference category = commercial test.
** Dummy variable, reference category = no training.
OR: Odds ratio.
CI : Confidence interval.

Discussion

The results of this study underscore that RSV testing at participating laboratories in eastern and western Europe is generally good, with exception of five laboratories that had a correct score of 50% to 70%. We have found a good performance of molecular assays for RSV-A detection, and a somewhat lower performance for RSV-B. To our knowledge, this is the first study that explores whether laboratory characteristics can predict the performance score. We found that the type of method (real-time PCR or nested PCR versus commercial test) was a significant factor in predicting a correct result. However, it is important to be aware of the risk for contamination problems in nested PCR.[20] In addition to the type of method, our study suggests that training and the use of a combination of target genes could also enhance the performance, even though results were not statistically significant. Repeating

this study in a large number of laboratories might result in the establishment of additional independent factors impacting the performance.

Possible reasons for a lower detection of RSV-B may be due to an actual lower sensitivity of the tests, which was observed for commercial and single PCR in particular. Commercial assays showed a lower performance, but since only three laboratories used this type of assay further research is required to investigate the real sensitivity of these and other commercial methods. A limitation of the study was the number of non-respondents; eight participants did not report results to QCMD. One of the laboratories withdrew because of inconclusive results and this may indicate problems in the testing and a bad performance. The results of the participating laboratories may therefore not be completely representative for all laboratories in Europe. Additionally, not all questions in the questionnaires were completed for all laboratories and these laboratories were therefore excluded from the multilevel analysis. The power of the study was determined by the number of participating laboratories, resulting in relatively wide confidence intervals in the multilevel analyses.

Another limitation of this study was that no clinical isolates were used and therefore only the technical sensitivity could be determined. Data on the absolute virus quantity in the samples were not available, only dilution factors, which were used as a semi-quantitative measure. The good results for in-house PCR tests probably reflect the good sensitivity of the test. However it may be possible that the primers of the molecular assays matched well with the ATCC strains of the panel, but whether this is also true for the actually circulating viruses that are present in clinical samples, could not be assessed in this study.

The majority of molecular assays that were available were research and clinical “in-house assays” utilizing PCR as the primary method of amplification. In our study, only three laboratories used a commercial test and these tests generally had a lower performance score than the in-house assays. A study carried out by Templeton et al.[10], included only one (other) commercial test for RSV, and had a correct score of 50%, which seems comparable to our results, but the number of commercial tests is small and the performance varies by type of test. Commercial tests generally performed well compared to in-house assays for other pathogens, as has been described for *Chlamydia trachomatis* and *Mycobacterium tuberculosis*, and for these pathogens the

number of laboratories that use commercial assays is substantial.[9,21] In our study, one false positive was detected in the negative sample, and this finding is similar to results from other quality control programs for virus detection.[10,11,22]

In terms of clinical implications, it is important that the diagnosis of RSV infection is made quickly. In practice quick RSV tests are also used and these have the advantage of having available results within 20-30 minutes. The sensitivity of these type of tests seem reasonable[23], but is lower when compared to other methods such as real-time and nested PCR.

This study has shown differences between laboratory characteristics, with only the type of assay explaining differences in test performance. The performance score for laboratories located in eastern Europe did not differ from the score of laboratories located in western Europe. Currently, many laboratories in eastern Europe carry out NATs, while molecular assays were not regularly performed five years ago.[24] Even though molecular assays are used for a few years in these laboratories they performed well in this external quality control assessment.

This study is the first step in providing insight in laboratory specific factors which can be used to improve the quality of RSV molecular diagnostics. The comparison of laboratory performance in diagnostic testing through external quality control studies is very important and allows the investigation of factors that affect the performance.

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7

Detection of multiple respiratory pathogens during primary respiratory infection: nasal swab versus nasopharyngeal aspirate using real-time polymerase chain reaction

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Abstract

In this study, we present the multiple detection of respiratory viruses in infants during primary respiratory illness, investigate the sensitivity of nasal swabs and nasopharyngeal aspirates and assess whether patient characteristics and viral load played a role in the sensitivity. Healthy infants were included at signs of first respiratory tract infection. Paired nasopharyngeal aspirates and nasal swabs were collected. Real-time polymerase chain reaction was carried out for eleven respiratory pathogens. Paired nasopharyngeal aspirates and nasal swabs were collected in 98 infants. Rhinovirus (n=67) and respiratory syncytial virus (n=39) were most frequently detected. Co-infection occurred in 48% (n=45) of the infants. The sensitivity of the nasal swab was lower than the nasopharyngeal aspirate in particular for respiratory syncytial virus (51% vs. 100%) and rhinovirus (75% vs. 97%). Sensitivity of the nasal swab was strongly determined by the cycle threshold value ($p<0.001$). Sensitivity of the swab for respiratory syncytial virus, but not rhinovirus, was 100% in children with severe symptoms (score≥11). It is concluded that for community based studies and surveillance purposes the nasal swab can be used, though the sensitivity is lower than the aspirate in particular for the detection of mild cases of RSV infection.

Background

Respiratory viruses are a common cause of illness in children, in particular during their first years of life and may lead to more severe morbidity and hospitalisation.[1-4] Different types of specimen are available for viral diagnosis. The nasopharyngeal aspirate (NPA) has been considered the best sampling technique, but is more invasive and results in significantly more distress of the infant than a nasal swab (NS).[5] A number of studies have compared the sensitivity of NPA with nasopharyngeal swabs[6], nose-throat swabs[7] and NS.[5,8-10] Generally conventional techniques such as viral culture and antigen detection methods were used. The use of real-time polymerase chain reaction (PCR) may overcome differences in sensitivity for respiratory viruses as a result of specimen type.[7]

Limited data is available on the comparison of these sampling methods combined with real-time PCR. It was observed that nose-throat swabs are a less invasive diagnostic technique with adequate sensitivity for use in outpatient and large community-based settings in children.[7] However, no tests were performed for rhinovirus, while this virus commonly infects infants.[11] The aim of this study was to present the detection of common respiratory pathogens in infants during primary respiratory illness, to investigate the sensitivity of NS and NPA, and assess the role of patient characteristics and viral load in the sensitivity of either sampling method.

Study Design

Study Cohort

The study is part of the Netherlands Amnion Fluid Study of the Utrecht University Medical Centre (UUMC), the Netherlands.[12] Healthy infants were included at birth, and were at risk for primary respiratory infection until the age of one year. The data collection and episode sampling stopped one year after birth. From April 2006 to April 2008, including two winter seasons, paired NPA and NS specimens were obtained from 98 infants. Parents were instructed to notify the clinical staff within 24 hours after onset of symptoms. Clinical staff visited the child within 36 hours and the history of illness was taken by a standardized questionnaire. Symptoms were scored, according to Gern[13], with points presented in parentheses: fever (> 38°C)(1); cough, mild(1), moderate(2), severe(3); rhinorrhea, mild(1), moderate to severe(2); hoarseness(1); duration of illness > 4 days(1); apnoea(3); wheezing(5); retractions(5); tachypnea(5); cyanosis(5). Mild, moderate and severe infection were defined as sum scores 0-4, 5-10 and 11 and higher, respectively. Specially trained clinical staff obtained paired NS and NPA.

Collection of specimens

The NPA was obtained by use of an infant mucus extractor (Vygon). Both nostrils were suctioned. In addition a NS was collected, samples were collected from one nostril and one from the hard palate using separate cotton-tipped swabs (Infant Mucus Extractor, Vygon Pharmaceutiques, Ecouen, France). The two swabs were then inserted in one vial containing 2 ml of virus transport medium (gly medium).

Real-time PCR

Semi-quantitative real-time PCR was conducted on both NS and NPA for RSV, rhinovirus, human metapneumovirus, adenovirus, coronavirus, influenza, parainfluenza virus type (type 1,3), parainfluenza (type 2,4), bocavirus, *Mycplasma pneumoniae,* and *Chlamydia pneumoniae*. Nucleic acids were extracted using the QIAamp DSP virus kit (QIAGEN, Valencia, CA). Each sample was eluted in 200 µl buffer. cDNA was synthesized by using MultiScribe reverse transcriptase (RT) and random hexamers (both from Applied Biosystems, Foster City, CA, USA).[14] Each 100 µl reaction mixture contained 60 µl cDNA mix and 40 µl of eluted RNA. After incubation for 10 minutes at 25°C, RT was carried out for 30 minutes at 48°C, followed by RT inactivation for 5 minutes at 95°C.[14] Samples were assayed in a 50 µl reaction mixture containing 20 µl (c)DNA, and 30 µl mix of the forward and reverse primers and probes. All samples had been spiked before extraction with an internal control virus (murine encephalomyocarditis virus [RNA] and phocine herpes virus [DNA]). The amplification and detection were performed by use of the ABI Prism 7700 sequence-detection system; 2 min at 50°C to acquire optimal AmpErase UNG activity and 10 min at 95°C to activate AmpliTaq Gold DNA polymerase, followed by 45 cycles of 15s at 95°C and 1 min at 60°C.[15] Primers and probes for real-time PCR detection of RSV, influenza virus, para-influenza virus and adenovirus are available by van de Pol et al.[16] and real-time PCR were performed as described previously[17-19].

Statistical analysis

Similar to previous studies, a consensus standard was used to assess sensitivity of each testing method: a positive result in either NPA or NS was considered as gold standard for presence of a pathogen and used to calculate the sensitivity of the NPA and NS for detection of the respiratory pathogens. Chi-square test was used and a logistic regression analysis was performed. The outcome variable was defined as the sample being positive. Variables inserted in the model were age, gender, symptom score, and multiple virus detection. Statistical significance was concluded if the p-value was <0.05. The statistical analyses were performed in STATA 10.0 (StataCorp LP, TX USA).

Results

A total of 163 respiratory pathogens were identified in 94 children. The majority of children (73.5%) were ill for less than 4 four days at the time of sampling. The median age at primary infection was 104 days (range 33-269) and the median score of symptoms was 3, indicating a mild illness (Table 7.1). In all children with an illness of five days or longer, one or more pathogen was detected.

Table 7.1: Characteristics of infants during first airway infection

Characteristics	All infants (n=98)	Rhinovirus detected (n=67)	RSV detected (n=39)
Median age in days (range)	104 (33-269)	99 (33-269)	115 (51-269)
Median days of illness at time sampling (range)	3.5 (2-31)[c]	4 (2-16)	4 (2-12)
% Male	58%	60%	62%
Symptoms			
Rhinorrhea			
None	15%	15%	18%
Mild	55%	58%	46%
Moderate-severe	30%	27%	36%
Cough			
None	17%	18%	5%
Mild	36%	46%	28%
Moderate	37%	30%	46%
Severe	10%	6%	21%
Wheezing [a]	8%	6%	10%
Fever >38°C	17%	12%	23%
Hoarseness	28%	22%	31%
Apnea [b]	3%	2%	5%
Cyanosis	1%	0%	3%
Retractions	6%	3%	5%
Tachypnea	20%	16%	28%
Median sumscore (range)	3 (0-25)	3 (0-15)	4 (1-25)
0-4	64%	72%	56%
5-10	25%	22%	26%
>10	11%	6%	18%

[a] reported by parents; [b] N=97, [c] duration of illness: IQR = 3-5.
Values represent percentages, unless indicated otherwise.

Multiple pathogens in half of the children

In 49 children (50%) one pathogen was detected: 29 rhinovirus, 11 RSV, 4 coronavirus, 2 hMPV, 2 parainfluenza virus type 2 and 4 and 1 bocavirus. In twenty-five children two pathogens were detected, of which 24 (96%) were rhinovirus with RSV. In 20 children more than two pathogens were detected during the first episode of respiratory symptoms (three pathogens: n=17; four pathogens: n=2; five pathogens: n=1). Co-infection rates by pathogen were: rhinovirus (57%), hMPV (60%), RSV (72%), coronavirus (71%) and bocavirus (91%).

Sensitivity of nasal swab is lower than the aspirate for RSV and rhinovirus

Rhinovirus was found most frequently (n=67), followed by RSV (n=39) and coronavirus (n=14) (Table 7.2). No influenza viruses and parainfluenza type 1 and 3 viruses were detected. Sensitivity for detecting any pathogen of the NPA was 92% ($CI_{95\%}$ 86.7-95.7), whereas the sensitivity of the NS was lower at 67% ($CI_{95\%}$ 59.1-74.0). For the detection of RSV and rhinovirus the sensitivity of the NS was lower than the NPA (Table 7.2).

Table 7.2: Detection of respiratory pathogens and the sensitivity by sampling method

Respiratory pathogen	NPA (n)	NPS (n)	Total	NPA		NS	
				Sensitivity	95%CI*	Sensitivity	95%CI*
Rhinovirus	65	50	67	97%	89.6-99.6	75%	62.5-84.4
RSV	39	20	39	100%	91.0-100	51%	34.8-67.6
Coronavirus	13	10	14	93%	66.1-99.8	71%	41.9-91.6
Bocavirus	8	7	11	73%	39.0-94.0	64%	30.8-89.1
Adenovirus	9	6	11	82%	48.2-97.7	55%	23.4-83.3
Parainfluenza type 2 and 4	9	9	11	82%	48.2-97.7	82%	48.2-97.7
hMPV	4	3	5	80%	28.4-99.5	60%	14.7-94.7
Mycoplasma Pneumoniae	2	2	3	67%	9.4-99.2	67%	9.4-99.2
Chlamydia Pneumoniae	1	2	2	50%	1.2-98.7	100%	15.8-100
Total	150	109	163	92%	86.7-95.7	67%	59.1-74.0

NPA: nasopharyngeal aspirate; NS: nasal swab; RSV: respiratory syncytial virus; hMPV: human metapneumovirus; CI: Confidence interval.
* One-sided 97.5% confidence interval was used in case sensitivity was 100%.

Sensitivity of nasal swab depends on viral load

Sensitivity values of the NPA and NS were investigated in more detail for rhinovirus and RSV (Table 7.3). For children with a low symptom score the sensitivity of the NS was lower than the NPA. The NS had a lower sensitivity than the NPA for the 30-40 CT values. To assess whether the sensitivity of the NS differed by age group, gender, multiple pathogens, symptom score and CT-value, chi-squared tests were performed. The sensitivity of the NS for detection of RSV was related to the symptom score ($p=0.001$), and the sensitivity of the NS was related to the CT values for both RSV and rhinovirus ($p<0.001$).

In the logistic regression analysis age and gender did not significantly predict the detection of RSV or rhinovirus. Symptom score predicted RSV detection in both the NPA (OR: 1.21; $CI_{95\%}$ 1.07-1.39) and the NS (OR: 1.28; $CI_{95\%}$ 1.12-1.48), while an inverse relationship was observed for symptom score and rhinovirus detection in the two samples (OR: 0.87; $CI_{95\%}$ 0.78-0.98). The presence of more than one pathogen predicted RSV (OR: 8.98; $CI_{95\%}$ 3.03-26.7) or rhinovirus detection (OR: 3.66 $CI_{95\%}$ 1.33-10.08) in the NPA. When the same analysis was performed as a backwards regression with $p<0.2$, results did not change.

Table 7.3: Sensitivity of the NPA and NS for the detection of rhinovirus and RSV presented by age group, gender, symptom score, presence of multiple pathogens and CT-value of the NPA

Respiratory pathogen	NPA (n)	NTS (n)	Total	NPA		NS	
				Sensitivity	95% CI*	Sensitivity	95% CI*
Rhinovirus							
Age							
1-3 months	28	24	28	100%	87.7-100	86%	67.3-96.0
3-6 months	30	22	32	94%	79.2-99.2	69%	50.0-83.9
6-12 months	7	4	7	100%	59.0-100	57%	18.4-90.1
Gender							
Boy	38	32	40	95%	83.1-99.4	80%	64.4-90.0
Girl	27	18	27	100%	82.1-100	67%	46.0-83.5
Symptom score							
0 to 4	46	36	48	96%	85.7-99.5	75%	60.4-86.4
5 to 10	15	11	15	100%	78.2-100	73%	44.9-92.2
over 11	4	3	4	100%	39.8-100	75%	19.4-99.4
Multiple pathogen							
No	29	25	29	100%	88.1-100	86%	68.3-96.1
Yes	36	25	38	95%	82.3-99.4	66%	48.7-80.4
CT NPA**							
0-20	3	3	3	100%	29.2-100	100%	29.2-100
20-25	22	22	22	100%	84.6-100	100%	84.6-100
25-30	17	15	17	100%	80.4-100	88%	63.6-98.5
30-35	10	6	10	100%	69.2-100	60%	26.2-87.8
35-40	10	2	10	100%	69.2-100	20%	2.5-55.6
40-45	3	0	3	100%	2.9-100	0%	0-70.8

Respiratory pathogen	NPA (n)	NTS (n)	Total	NPA		NS	
				Sensitivity	95% CI*	Sensitivity	95% CI*
RSV							
Age							
1-3 months	12	7	12	100%	73.5-100	58%	27.7-84.8
3-6 months	20	11	20	100%	82.3-100	55%	31.5-77.0
6-12 months	7	2	7	100%	59.0-100	29%	7.6-64.8
Gender							
Boy	24	11	24	100%	85.8-100	46%	25.6-67.2
Girl	15	9	15	100%	78.2-100	60%	32.3-83.4
Symptom score							
0 to 4	22	8	22	100%	84.6-100	36%	17.2-59.3
5 to 10	10	5	10	100%	69.2-100	50%	18.7-81.3
over 11	7	7	7	100%	59.0-100	100%	59.0-100
Multiple pathogen							
No	11	10	11	100%	71.5-100	91%	58.7-99.8
Yes	28	10	28	100%	87.7-100	36%	18.6-56.0
CT NPA**							
0-20	4	4	4	100%	39.8-100	100%	39.8-100
20-25	11	10	11	100%	71.5-100	91%	58.7-99.8
25-30	4	4	4	100%	39.8-100	100%	39.8-100
30-35	7	1	7	100%	59.0-100	14%	0.4-57.9
35-40	10	0	10	100%	69.2-100	0%	0-30.8
40-45	3	1	3	100%	29.2-100	33%	0.8-90.6

NPA: Nasopharyngeal aspirate; NS: nasal swab; RSV: respiratory syncytial virus; CT: Cycle threshold value.
* One-sided 97.5% confidence interval was used in case sensitivity was 100%.
** The CT-value of the NPA was used as a reference to compare with NS, therefore the sensitivity of the NPA is 100% for all categories.

Discussion

This study presents that the proportion of infants where a respiratory pathogen detected was high (96%), and co-infections were common. In twenty children more than two pathogens were detected during the first episode of respiratory symptoms. Co-infections were observed frequently for RSV (72%), coronovirus (71%) and bocavirus (91%) in particular.

High rates of co-infection in young children have been described recently for childhood pneumonia in particular in children aged less than 12 months[20], and in children hospitalized with acute respiratory tract infection.[21,22] The most frequently detected virus was RSV followed by human bocavirus, and rhinovirus.[21,23] A common combination has been reported to be RSV and bocavirus.[21] Even though high occurrence of co-infections has been reported, ranging from 14-16%[21,22] to 27%[20], our study presents an even higher rate of co-infection. A possible explanation for this high co-infection rate may be related to the sampling of both nostrils for the NPA. Human bocavirus is a newly identified virus and has been detected in respiratory tract secretions in patients with acute respiratory symptoms in 2 to 19% of the samples.[24] Co-infection with another virus has been observed in 40% of the bocavirus-positive children.[25] The frequent associations of bocavirus with other respiratory viruses might be explained by the persistence of bocavirus in the respiratory tract.[25]

Furthermore, we investigated the sensitivity of NPA and NS tested by a real-time PCR method. The sensitivity of the NPA was 92%, while for the NS this was 67%. In particular for the detection of rhinovirus and RSV, the NS had a lower sensitivity (75% and 51% respectively) compared to the NPA (97% and 100%). Sensitivity of the NS for RSV was 100% for children with high symptom scores. For both RSV and rhinovirus, viral load, indicated by the CT value, was the major determinant of sensitivity of NS in a dose-dependent fashion. Symptom score predicted RSV detection in both the NPA and the NS, while an inverse relationship was observed for symptom score and rhinovirus detection in the two samples.

The use of a swab has been considered as a suitable replacement in community-based research or epidemiological studies. The major advantage of a swab is that collection is less painful, and more convenient than an aspirate

as no additional devices are needed.[5] These factors may outweigh some reduction in sensitivity. The advantage of molecular methods in the detection of respiratory viruses has been reported[26,27] and Lambert et al. reported that using these methods seemed to overcome the previously observed sensitivity reduction when less invasive specimens were combined with the conventional laboratory methods.[7] With the recently developed flocked swabs, sensitivity is even further improved and the flocked swabs have the advantage of being rapid, and less traumatic for paediatric patients.[28] However, the sensitivity of the flocked swab in outpatient respiratory tract infection may be lower than in hospitalized patients. Further studies are required considering different types of swabs and patient populations, and should test for a broad spectrum of respiratory pathogens.

Our findings demonstrated a lower sensitivity of the NS, in particular for RSV. Similar results were reported in other studies where conventional, non-amplification based-methods were used.[8,9] Lambert et al. did not test for rhinovirus, and this was the most frequently detected virus in our study and elsewhere.[11,29] No influenza detections were found in our study. This is not explained by sampling bias, because most swabs were taken during the winter season, during which both RSV and influenza had their peak incidence. A possible reason may be related to the patient population and the small population size. The study performed by Bueno *et al*. showed similar results with rhinovirus and RSV being most frequently detected.[29]

There were a number of limitations of this study. Firstly, one limitation was the timing of sampling. For five cases sampling occurred 10 days after the onset of illness. Since viral shedding of RSV is highest between days 0 and 6 sampling should preferably occur in this period.[30] The high proportion of positive samples however indicates this effect was not a major drawback of this study. Secondly, in this study pain and discomfort of the collection of the samples was not assessed, but other studies provided reference for this.[5] Finally, it is unknown whether the order of obtaining the specimens may have resulted in a lower detection rate in NS. It is possible that by suctioning both nostrils for the NPA the secretions with virus or viral nucleic acids were reduced. This corresponds with the finding that few mild cases were detected with the NS, as sensitivity of the NS dropped with lower symptoms score and higher CT values. Because the order and nature of the sample collection was slightly different from the study performed by Lambert et al., the sensitivity of

the NS may be an underestimate, and caution needs to be taken when interpreting the NS sensitivity.

RSV and rhinovirus were commonly detected in infants during primary respiratory infection, and co-infections occurred in about half of the children. The sensitivity of NPA was higher than NS, in particular for detection of RSV and rhinovirus. Although sensitivity of a method is important, one must also take into account the advantages that different sampling methods offer. The great advantage of the NS is that this method can be performed in outpatient settings without needing special devices, is less costly and causes less distress in the patient than the NPA. Although there is a reduction in sensitivity for RSV particularly in infants with mild symptoms, the NS is convenient for sampling patients in community studies and can be used for surveillance purposes.

Acknowledgement and Conflicts of Interest

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The medical ethical committee of the UUMC approved the study protocol and written informed consent was obtained from the parents of the participating children.

The authors declare that they have no conflicting interests in publishing this paper.

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8

General Discussion

Summary and general discussion

This final chapter provides an overview of findings presented in this thesis, methodological considerations, the implications of the findings and recommendations for future research.

The aim of this thesis was to improve surveillance and diagnostic methods of respiratory syncytial virus (RSV) in Europe. We have investigated:
a. which countries collect RSV data and whether these data are being reported timely,
b. the occurrence of RSV and the relation with meteorological factors,
c. what diagnostic methods are used to detect RSV, and
d. what factors relate to the sensitivity of a diagnostic RSV test.

Summary of the main findings

Surveillance of RSV

In Chapter 2 we reported on an investigation of the virological data collection for influenza and whether RSV data were collected in 19 countries[1]. This study was carried out in June 2002. We have found that the laboratory techniques for the detection of influenza were heterogeneous, and specimen collection and transport procedures were similar. Most national influenza surveillance networks collected nasal and throat swabs and ELISA, HAI and PCR were generally used for typing and subtyping of influenza viruses. In Western Europe PCR was regularly used, while in Eastern Europe, ELISA was predominantly used. Thirteen out of nineteen countries tested the specimens for RSV as well and some for other respiratory viruses. The substantial number of countries that tested for RSV indicates that more detailed information on RSV activity in Europe can be provided.

In Chapter 3 we presented the data reporting and timeliness of influenza and RSV data for three countries in Europe.[2] Timely data are important in order to function as an early warning system for outbreaks of respiratory disease. Data on both RSV and influenza were provided timely (i.e. within 1 week after detection) and country-specific data showed that RSV contributed considerably to the burden of influenza-like illness in particular in the United Kingdom. We

formulated the following recommendations for RSV surveillance: 1) Specimens collected as part of an influenza surveillance programme should also be tested for RSV; 2) Both combined nose-throat swabs and nasopharyngeal aspirates are acceptable for RSV diagnostics; 3) The application of molecular techniques such as real-time PCR in the diagnosis of respiratory disease has been demonstrated and we advocate this technique for RSV detection; 4) Further develop standardized methods and laboratory techniques; 5) Consider the development of a sentinel approach of representative hospitals; 6) Integration of RSV surveillance in countries joining the surveillance system alongside influenza.

Subsequently we presented the progress in the surveillance of RSV in Europe over the period 2001-2008 in Chapter 4.[3] In 2003, an RSV Task Group was established within the surveillance network to explore the possibility to design a comprehensive RSV surveillance scheme. In this Chapter we present steps forward that were made for the recommendations that were introduced in Chapter 3. We found that the number of European countries testing specimens for RSV increased from six to fourteen and respiratory specimens from nose and/or throat were generally used for detection of influenza and RSV. A total of 25 laboratories performed molecular testing for diagnosing RSV and participated in a quality control assessment for RSV with an overall good performance.[4] Limited progress was achieved for standardizing methods and for the development of a sentinel surveillance system for RSV of representative hospitals. Four out of ten new countries started reporting RSV detections in addition to influenza in the period 2004-08.

Seasonal variation of RSV and the role of the weather conditions

The seasonal pattern of RSV and the role of the weather conditions in the Netherlands was investigated and presented in Chapter 5. We found that the onset of RSV activity occurred around week 44, peaked around week 52, and was quite consistent for the study period (8 winters). This information is useful for the timing of the palivizumab antibody prophylaxis. The minimum temperature was variable for the different winter seasons but a generally lower temperature was observed around the peak activity. The relative humidity, minimum temperature, and cloud cover were significantly correlated with the weekly number of RSV cases. This finding was observed for different time lags, but the r-value decreased with increasing time lag for relative humidity and cloud cover.

In multiple linear regression analysis at least one of the meteorological factors, minimum temperature, relative humidity and cloud cover, was significantly associated with RSV activity in seven out of eight winters seasons. For all seasons together, the relation of temperature with RSV activity was negative, indicating more RSV activity when the temperature decreased. A positive relation was observed for relative humidity and to some extent for cloud cover, indicating more RSV activity when the relative humidity or cloud cover increased. Our data showed that relative humidity, minimum temperature and cloud cover were important predictors of RSV activity for different time lags in the Netherlands, with the effect of relative humidity being most consistent.

Laboratory testing for RSV

Chapter 6 presents the findings of a European quality control study for RSV detection and highlights the impact of laboratory characteristics.[4] A panel consisting of nine coded samples containing RSV-A and /or RSV B and one negative sample was evaluated in 25 laboratories across Europe. The overall mean percentage of correct results was 88%, and ranged from 50% to 100%. The percentage correct results decreased in correspondence with decreasing sample concentration. The type of assay (nested or real-time PCR vs. commercial tests) was identified as a significant factor (OR 8.39; $CI_{95\%}$ 1.91-36.78) in predicting a correct result, which indicates that nested or real-time PCR performed better than the commercial assays.

In Chapter 7 we reported on a study that assessed the sensitivity of real-time PCR for eleven respiratory pathogens in two types of specimens. Healthy infants were included at signs of first respiratory tract infection. The study period was from April 2006 to April 2008 including two winter seasons. Paired nasopharyngeal and nasal swabs were collected in 98 infants. In 94 infants one or more pathogen was detected. Rhinovirus was found most frequently (n=67), followed by RSV (n=39) and coronavirus (n=14). The overall sensitivity of the nasopharyngeal aspirate was 92%, and for the nasal swab the sensitivity was 67%. The sensitivity of the nasal swab was similar or higher to the nasopharyngeal aspirate for parainfluenza type 2 and 4, *Mycoplasma Pneumoniae* and *Chlamydia Pneumoniae*. The nasal swab performed less well than the nasopharyngeal aspirate for the other respiratory pathogens, with the difference for RSV and rhinovirus being most distinct. For both RSV and rhinovirus detection the viral load, indicated by the CT-value, was the major

determinant of sensitivity of the nasal swabs. Age and gender did not predict the detection of RSV or rhinovirus. A higher symptom score was related to RSV detection in both the nasopharyngeal aspirate and nasal swab, and a lower score was related to rhinovirus detection.

Methodological considerations

The Chapters in this thesis are based on different sources of data. The surveillance-related data (Chapters 2-4) were descriptive and largely based on sentinel surveillance data. In Chapter 5, weekly RSV cases reported by the Infectious diseases Surveillance Information System (ISIS) were used and weather data was obtained from the Royal Netherlands Meteorological Institute (KNMI). Chapter 6 described a quality control assessment for the detection of RSV for 25 laboratories and assessed laboratory characteristics as determinant of outcome of the test. Finally, in Chapter 7 real-time PCR methods were used to detect respiratory pathogens in infants during primary respiratory infection, using two types of respiratory specimens.

Limitations methodology

We will first describe the limitations concerning data obtained by surveillance. Surveillance is used to provide an early warning function or outbreak detection. The basis of the surveillance platform presented in this thesis is integrated clinical and virological data collection from sentinel GPs. In sentinel surveillance, standard case definitions and protocols must be used to ensure validity of comparisons across time and sites despite lack of statistically valid sampling.[5] Although the European Influenza Surveillance System was established in 1996 and has made efforts in harmonizing the surveillance, the case definitions used by individual countries are heterogeneous and methodologies differ somewhat by country.[6] This is one limitation that makes between-country comparisons difficult.

Another important limitation concerns the fact that an influenza surveillance network was used to report data on RSV. The case definitions for influenza-like illness and acute respiratory illness have been established for influenza and not for RSV. But since RSV infection may be difficult to distinguish from influenza[7], the influenza-case definitions may also be applicable for RSV as well, as long as no RSV-specific definition has been defined. Anyway, it is difficult to apply rigid guidelines across all age groups and patients that consult in different phases in their illness.[8]

In particular young children, aged 0-4 years, visit their general practitioner for respiratory complaints. However, the sampling of these patients may actually be underrepresented in some countries[2], as doctors may be reluctant to swab infants and regular swabs may not be suitable for small children. On the other hand, non-sentinel hospitals predominantly report the detection of RSV in young children. One needs to take this into account when interpreting the RSV data from different sources.

For the study on the role of meteorological factors with RSV, some limitations can be posed. Data were available for the Southern and Eastern parts of the Netherlands. Additionally, the number of laboratories differed by season and the weather data were collected from one central point in the country. Furthermore, the effect of meteorological factors was less obvious when data were assessed by individual winter season, but this may be due to the low number of RSV cases reported in some winters. It would be interesting to investigate whether the contribution of temperature and humidity to RSV activity can also be observed for other countries in Europe.

Limitations for the quality control study for RSV are: 1) the number of participating laboratories, with 25 out of 33 laboratories participating, and 2) the use of ATCC strains instead of clinical samples. Data on the absolute virus quantity in the samples were not available, only dilution factors, which were used as a semi-quantitative measure. However, the good results for in-house PCR tests probably reflect the good sensitivity of the test. Finally, in Chapter 7 we compared the sensitivity of molecular assays for two types of respiratory specimens. We observed a very high percentage of positive specimens (96%), and a co-infection rate of 48%. While it is important for a diagnostic test to have a high sensitivity, the most recent PCR techniques may also pick up irrelevant viral infections. With multiple viral pathogens detected, the aetiology of respiratory illness is troubled.

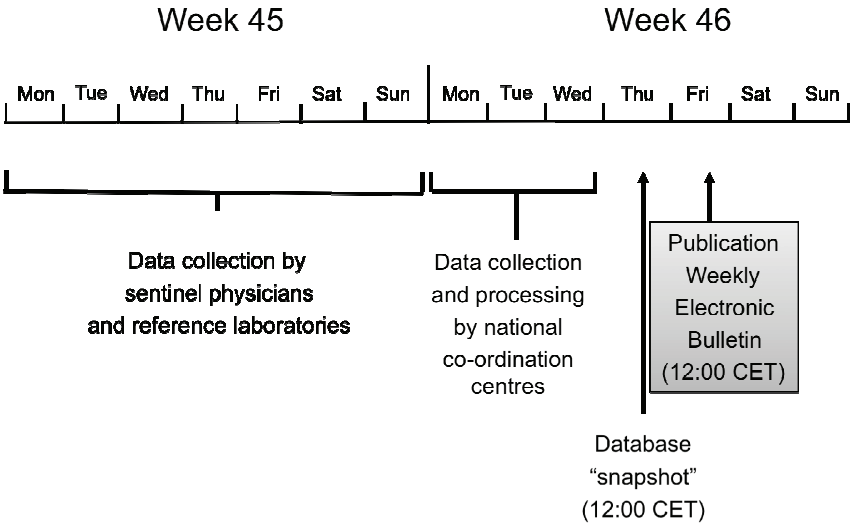
Strengths surveillance

One of the strengths of influenza and RSV surveillance described in this thesis is the integrated clinical and virological surveillance model.[9] In this model sentinel general practitioners usually covering 1-5% of the population report on the occurrence of influenza-like illness and/or acute respiratory infection. From a proportion of these patients a respiratory specimen is obtained and sent to the laboratory for diagnosis. A great advantage of this sentinel

surveillance system is the availability of a population denominator which allows to present age-specific rates of influenza-like illness and/or acute respiratory infection. The large number of countries that have been working together and sharing information allowed to present information on the circulation of influenza and RSV in a large number of European countries.[10]

In addition to the integrated sentinel data, non-sentinel data are collected for surveillance. The non-sentinel data are generally derived from hospitals. The combination of the two data sources provides a full picture of respiratory infections in both the community and the hospital setting. Another strength of surveillance is the timeliness of the data collection. Countries enter surveillance data into an internet-based platform since 1996.[11] Data on influenza and RSV are timely published in a weekly electronic bulletin with a delay of one week (Figure 8.1). Surveillance of both influenza and RSV, which circulate in the community around the same time of the year, facilitates to unravel the impact of these respiratory viruses.

Figure 8.1: Data reporting and data presentation in a weekly electronic Bulletin.



Implications of the findings

Monitoring respiratory syncytial virus

Effective surveillance is essential to keep on top of winter illnesses and routine respiratory virus surveillance can predict their arrival, monitor their progression and recommend appropriate advice on how to contain them.[8] For example, preventive measures can be taken and/or evaluated in neonatal wards and the RSV surveillance can be useful for the timing of palivizumab prophylaxis. In the US, regional RSV surveillance has been already implemented to support clinical decision making for prophylaxis in premature infants[12,13] and the data obtained through RSV surveillance has been useful in understanding the seasonal and geographical RSV trends.

Laboratory diagnosis and clinical management

By performing laboratory testing we can elucidate the aetiology of infection. The information can be used to prevent further infection in children at risk, for example by isolating RSV-infected infants at the neonatal care unit. Recent advances in laboratory techniques have provided more sensitive methods of detection in comparison to "gold standard" detection methods as virus culture. With new methods such as real-time PCR, diagnosis and the quality of surveillance are improved. However, our results presented in Chapter 7, provided a very high proportion of positive specimens (96%) and in about half of the children more than one pathogen was detected. This may indicate that the PCR may have picked up irrelevant infections for the clinical diagnosis and makes it difficult to determine which pathogen was the main cause of the respiratory illness.

Although antibiotics have little or no benefit for colds and upper respiratory tract infections, these conditions account for a sizable proportion of antibiotic prescriptions.[14] It has been hypothesized that quick laboratory tests results presenting a viral cause of the illness may lead to a reduction in antibiotic use. A number of studies have investigated the effect of rapid diagnostic tests on antibiotic use, but the results are inconclusive[15-19]; it may be that different effects are observed for different age groups and are related to the severity of illness. One difficulty in the hypothesis is that detection of a virus does not exclude the presence of a bacterial pathogen. For example studies of autopsy specimens have shown that 22 (29%) of the 77 patients attributed to pandemic influenza A (H1N1) 2009 virus had molecular evidence of co-infection with an

identified bacteria, including 10 persons infected by *Streptococcus Pneumoniae*.[20] Therefore, in the diagnosis of respiratory illness it is important to test for both viral and bacterial pathogens.

Vaccination
Currently, no licensed RSV vaccine is available. Several factors have interfered with the development of an RSV vaccine. Firstly, infants who are at greatest risk of severe disease have a weak immune response. In addition, circulating maternally derived antibody may interfere with the immune response of the infant. Another important point is related to the testing of a formalin-inactivated RSV vaccine in infants and children in the 1960s. The vaccine did not protect against infection and was associated with an increased risk of severe RSV disease when some of the vaccinated children became naturally infected.

Two types of candidate RSV vaccines are being evaluated in humans: live attenuated vaccines and sub-unit vaccines. An RSV immunization program may need to include different vaccines for the different target groups such as infants in the first year of life, expectant mothers (so the mother will pass immunity to her baby), and high risk groups.[21] Maternal vaccination may be an interesting strategy and has the potential for preventing RSV disease in early infancy. However, for safety reasons, researchers have intuitively been reluctant to administer an RSV vaccine to pregnant women.[22]

In the light of a future vaccine, surveillance of RSV and the application of sensitive diagnostic methods are highly relevant. There are three important variables in designing efficacy trials: 1) age at vaccination, 2) clinical endpoints and 3) laboratory diagnosis. Age-specific rates of acute respiratory illness, lower respiratory tract infection and hospitalization may be used as clinical endpoints for a vaccine efficacy trial.[23]

Recommendations for future research

Currently, aggregated data form the basis of the surveillance. Limited data is available on the number of hospitalised patients and number of deaths. Quality of surveillance would improve if information on related illnesses, antibiotic use, hospitalisation etc. are collected. There are two points that need

attention to accomplish this: firstly case-based information derived from patient consultation and investigation should be made available. This however may interfere with confidentiality and ethical issues[8]. Secondly, the required surveillance data (ILI, laboratory diagnosis, hospitalisation, treatment, underlying disease) originate from separate sources. Therefore, we would recommend to link information between the different sources of data. The power of surveillance would greatly be enhanced by linking different data sources and establishing a fully integrated surveillance model from clinical diagnosis to hospitalisation. With modern information technologies this becomes more feasible.

Within general practice, consultations for acute respiratory infection are common. In children 0-4 years old, infection by RSV is probably the major cause of acute bronchiolitis; in the elderly RSV can also cause a significant burden[24,25], but limited data are available on the role of RSV in hospital admissions and deaths in the elderly. The highest proportions of respiratory admissions are limited to the young and old, and respiratory death are limited almost exclusively to the elderly[8]. Therefore, it is important to monitor respiratory illness in all age groups in both the community and the hospital setting. Implementing surveillance in the frail elderly in nursing homes could be a major step forward in improving surveillance. For future research we would recommend to perform a prospective cohort study to investigate the impact of respiratory pathogens, including RSV, in a nursing home setting and investigate viral shedding in elderly patients. This will provide more insight in the incidence and the respiratory burden in general in this population.

In the United States, the New Vaccine Surveillance Network was established in 1999 to evaluate the impact on new vaccines and vaccine policies through active sentinel surveillance. At three medical centers population-based inpatient and enhanced outpatient surveillance for vaccine-preventable diseases is conducted. The program complements existing surveillance programs for vaccine-preventable diseases by conducting seasonal active population-based surveillance for hospitalizations associated with acute respiratory illness.[26] A similar network could be implemented in Europe, with taking the influenza surveillance presented in this thesis as framework.

Recent developments in surveillance indicate that also syndromic surveillance can be used to detect respiratory virus activity.[27,28] For sampling of patients, self-sampling may be a good alternative, as it seems a feasible method of enhancing community-based surveillance programmes for detection of influenza.[28] In addition internet-based monitoring has been performed in some European countries and showed that reliable data can be collected.[29] All these types of data can be used to further enhance the surveillance of respiratory pathogens.

Conclusions

The importance of RSV surveillance needs to be better recognised. Information derived from surveillance can be used to understand outbreaks and identify causative pathogens and the extent of spread into the population.

With the research presented in this thesis, we have demonstrated that the methods of surveillance have been improved; many countries have started reporting data on RSV, and data were reported timely. For the Netherlands, we have found a regular onset of RSV activity around week 44 and a peak over the Christmas holidays. A regional surveillance of RSV would be useful to develop in European countries, as this would help in defining the timing of palivizumab prophylaxis. Furthermore, we have demonstrated that many countries use molecular assays for the detection of RSV and a good performance was observed for nested and real-time PCR in particular. Finally, multiple respiratory specimens were detected in infants during primary respiratory infection. Nasopharyngeal aspirates were superior to nasal swabs in terms of sensitivity for RSV and rhinovirus detection.

The findings presented in this thesis provide reference to further establish surveillance of RSV on both a regional and European level. RSV surveillance is relevant for providing knowledge on “who” is infected and “when”, and will provide useful information for the timing of administration of palivizumab. In addition, in the light of a future vaccine, fully integrated surveillance data - from clinical diagnosis to hospitalisation - is important and may be used to assess vaccine efficacy.

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Summary

Summary

Chapter 1 is a general introduction to this thesis. Respiratory syncytial virus (RSV) causes a substantial burden in particular in the very young and old. The spectrum of clinical disease ranges from mild upper respiratory tract illness, otitis media, croup to apnea in premature infants, pneumonia and bronchiolitis. In Europe, RSV accounts for 42-45% of hospitals admissions for lower respiratory tract infections in children younger than two years of age. At present there is no licensed RSV vaccine. Profylactic use of neutralizing antibody is available. Monthly administration of palivizumab reduces the risk of hospitalisation in premature infants and infants with chronic lung disease or congenital heart disease. Currently, rapid and sensitive molecular techniques for RSV diagnosis are available. With surveillance of RSV, outbreaks can be rapidly detected. In addition, the longitudinal surveillance data can be used to measure the impact of palivizumab prophylaxis and effectiveness of a future RSV vaccine. In this thesis we aimed to improve surveillance and diagnostic methods of respiratory syncytial virus in Europe. In **Chapter 2** we have assessed the comparability of virological data for the detection of influenza in countries in Europe and investigated which countries collect RSV data. The type of respiratory specimen and the transport conditions were similar. The diagnostic methods were diverse, and PCR was more often carried out in countries in Western Europe. In **Chapter 3** we assessed whether data on RSV collected by a European influenza surveillance network could be used to build an RSV surveillance system in Europe. Data on RSV from France, the Netherlands, England and Scotland were used. The data were entered timely. RSV contributed noticeably to influenza-like illness. Recommendations for RSV surveillance were formulated: 1) Specimens collected as part of an influenza surveillance program should also be tested for RSV; 2) Both combined nose-throat swabs and nasopharyngeal aspirates are acceptable for RSV diagnostics; 3) The application of molecular techniques such as real-time PCR in the diagnosis of respiratory disease has been demonstrated and we advocate this technique for RSV detection; 4) Further develop standardized methods and laboratory techniques; 5) Consider the development of a sentinel approach of representative hospitals; 6) Integration of RSV surveillance in countries joining the surveillance system alongside influenza. Following the surveillance recommendations, we presented the progress over seven years (2001-2008) (**Chapter 4)**. By 2008, progress was made for four out of six recommendations: the number of European countries testing specimens for RSV increased from six

to fourteen; nose and/or throat swabs were generally used for detection of influenza and RSV; a total of 25 laboratories performed molecular testing for diagnosis and participated in a quality control assessment for RSV with an overall good performance; four of the ten countries that joined EISS in 2004 started reporting RSV detections in addition to influenza in the period 2004-8. Limited progress was achieved for standardising methods and the development of a sentinel surveillance system of representative hospitals. In **Chapter 5** we have described the seasonal variation in RSV activity and investigated which meteorological variables are related to RSV outbreaks for different time lags. Time lags up to 4 weeks were included to assess a possible delayed weather effect in relation to RSV activity. We have found that the onset of RSV activity occurred around week 44 and activity peaked around week 52. Relative humidity was positively associated with RSV activity for all time lags, indicating more RSV when relative humidity increased. Minimum temperature was negatively associated with RSV activity and cloud cover was positively related with RSV activity. Relative humidity, minimum temperature, and cloud cover are important predictors of RSV activity in the Netherlands, with the effect of relative humidity being most consistent. In **Chapter 6** the laboratory performance of nucleic acid amplification techniques for respiratory syncytial virus (RSV) diagnosis was investigated in 25 laboratories across Europe. In addition we explored what factors were related to the diagnostic performance. The overall sensitivity for all laboratories was 88% (n=25; range 50-100). A correct score of 93% (range 70-100) was observed for laboratories performing in-house real-time PCR or nested PCR. Multilevel analysis showed that the type of assay (nested or real-time PCR vs. commercial test) was a significant factor (OR=8.39; $CI_{95\%}$ 1.91-36.78) in predicting a correct result. The results for this external quality control study showed that the overall performance of laboratories for RSV diagnosis in Europe is good and that real-time PCR is preferably used for RSV diagnostics. In **Chapter 7** we present the detection of respiratory viruses in infants during primary respiratory illness, investigate the sensitivity of nasal swabs and nasopharyngeal aspirates and assess whether patient characteristics and viral load played a role in the sensitivity. Paired nasopharyngeal aspirates and nasal swabs were collected in 98 infants. Rhinovirus (n=67) and respiratory syncytial virus (n=39) were most frequently detected. Co-infection occurred in 48% (n=45) of the infants. The sensitivity of the nasal swab was lower than the nasopharyngeal aspirate in particular for respiratory syncytial virus (51% vs. 100%) and rhinovirus (75% vs. 97%). Sensitivity of the nasal swab was strongly determined by the cycle threshold

value ($p<0.001$). Sensitivity of the swab for respiratory syncytial virus, but not rhinovirus, was 100% in children with severe symptoms (score≥11). It is concluded that for community based studies and surveillance purposes the nasal swab can be used, though the sensitivity is lower than the aspirate in particular for the detection of mild cases of RSV infection. Finally, in **Chapter 8**, the results of the earlier chapters were summarised and discussed and the implications and recommendations for future research were formulated. RSV surveillance may be used to support clinical decision making for prophylaxis in premature infants and the data obtained through RSV surveillance has been useful in understanding the seasonal and geographical RSV trends. The findings of this thesis will provide reference to further establish surveillance of RSV on both a regional and European level. RSV surveillance is relevant for providing knowledge on "who" is infected and "when", and will provide useful information for the timing of administration of palivizumab. In addition, in the light of a future vaccine, fully integrated surveillance data - from clinical diagnosis to hospitalisation - is important and may be used to assess vaccine efficacy.

Samenvatting

Samenvatting

In **hoofdstuk 1** wordt een algemene inleiding gegeven van dit proefschrift, getiteld Respiratoir syncytieel virus: het verbeteren van surveillance en diagnostiek in Europa. Het respiratoir syncytieel virus (RS-virus) infecteert de luchtwegen en veroorzaakt een aanzienlijke ziektelast in voornamelijk jonge kinderen en ouderen. Het klinische beeld varieert van milde bovenste luchtweginfectie, otitis media, kroep en apneu in prematuren, tot longontsteking en bronchiolitis. Een groot deel van de ziekenhuisopnamen voor lagere luchtweginfecties in kinderen onder de twee jaar is gerelateerd aan infectie met het RS-virus. Tot op heden is er geen vaccin tegen RS-virus infectie. Wel is profylactisch gebruik van neutraliserende antilichamen (palivizumab) beschikbaar. De maandelijkse toediening van palivizumab vermindert het risico op ziekenhuisopname in prematuren en kinderen met een chronische longaandoening of een aangeboren hartafwijking. Tegenwoordig zijn er snelle en zeer gevoelige moleculaire technieken beschikbaar voor het aantonen van het RS-virus. Door het monitoren van het RS-virus kunnen uitbraken van dit virus snel worden gedetecteerd. Daarnaast kan longitudinale surveillance data worden gebruikt om het effect van palivizumab profylaxe te meten. Op langere termijn zal surveillance data ook nuttig zijn om inzicht te krijgen in de effectiviteit van een toekomstig vaccin. Het doel van dit proefschrift is het verbeteren van surveillance en diagnostische methoden voor detectie van het RS-virus in Europa. In **Hoofdstuk 2** is onderzocht hoe vergelijkbaar de influenza dataverzameling is in landen binnen Europa. Tevens hebben we onderzocht welke landen testen uitvoeren om het RS-virus en andere respiratoire pathogenen te detecteren. In de meeste landen werden neus en/of keelwatten afgenomen. De transportomstandigheden waren vergelijkbaar. Verschillende diagnostische methoden werden gebruikt; polymerase keten reactie (PCR) werd vooral gebruikt in de landen in West-Europa. In **Hoofdstuk 3** is onderzocht of RS-virus data verzameld via een Europees influenza surveillance netwerk bruikbaar was voor het opzetten van RS-virus surveillance. RS-virus detecties in Frankrijk, Nederland, Engeland en Schotland werden beschreven. De data werden tijdig ingevoerd. Het RS-virus werd regelmatig aangetoond in patiënten met een griepachtig ziektebeeld. Tevens werden de volgende aanbevelingen gedaan:

1. Neus- en keelwatten die worden verzameld ten behoeve van influenza surveillance zouden ook getest moeten op aanwezigheid van het RS-virus;
2. Zowel gecombineerde neus-keelwatten en nasopharyngeale aspiraten zijn

acceptabel voor detectie van het RS-virus;
3. Het gebruik van moleculaire technieken zoals real-time wordt aanbevolen voor RS-virus detectie;
4. Verdere ontwikkeling van gestandaardiseerde methoden en laboratorium-technieken;
5. De ontwikkeling van een "sentinel" benadering van representatieve ziekenhuizen;
6. Integratie van RS-virus surveillance naast influenza in nieuwe deelnemende landen binnen het influenza surveillance systeem.

De vooruitgang van RS-virus surveillance binnen het influenza surveillance systeem over een periode van 7 jaar (2001-2008) is beschreven in **Hoofdstuk 4**. Er is vooruitgang geboekt voor vier van de zes aanbevelingen gepresenteerd in Hoofdstuk 3: 1) het aantal Europese landen dat neus- en/of keelwatten testte voor het RS-virus nam toe van zes naar veertien; 2) neus- en/of keelwatten werden doorgaans gebruikt voor de detectie van influenza en het RS-virus; 3) 25 laboratoria hebben deelgenomen aan een kwaliteitscontrole voor moleculaire technieken. De laboratoria hadden een goede score; 4) vier van de 10 nieuwe landen rapporteerden RS-virus detecties naast influenza virus detecties in de periode 2004-2008. Beperkte vooruitgang was geboekt in het standaardiseren van methoden en de ontwikkeling van een "sentinel" surveillance systeem van representatieve ziekenhuizen. In **Hoofdstuk 5** is de seizoensvariatie van het RS-virus beschreven en we hebben onderzocht welke meteorologische factoren zijn gerelateerd aan RS-virus activiteit. Om te onderzoeken of er een mogelijk verlaat effect van weersfactoren was op RS-virus activiteit zijn verschillende tijdsmomenten meegenomen in de analyses, deze varieerde van 0 tot 4 weken. De start van RS-virus activiteit was rond week 44 en de activiteit was het hoogst rond week 52. De relatieve vochtigheid was positief geassocieerd met RS-virus activiteit, dit houdt in dat bij er meer RS-virus detecties waren bij een hogere relatieve vochtigheid. Minimum temperatuur was negatief geassocieerd met RS-virus activiteit - oftewel er waren meer RS-virus detecties bij een lagere temperatuur. Tevens was de bewolkingsgraad positief geassocieerd met RS-virus activiteit. Relatieve vochtigheid, minimum temperatuur, en de bewolkingsgraad zijn belangrijke predictoren van RS-virus activiteit in Nederland. In **Hoofdstuk 6** is voor 25 laboratoria in Europa de kwaliteit van de nieuwste moleculaire technieken voor de detectie van het RS-virus onderzocht. Daarnaast hebben we onderzocht welke factoren gerelateerd waren met de sensitiviteit van de test. De sensitiviteit voor testen voor alle laboratoria was 88% (n=25). Een correcte

score van 93% vonden we voor laboratoria die een in-house real-time PCR of nested PCR gebruikten. Multilevel analyse liet zien dat het type test (nested of real-time PCR vs. commerciële test) een significante factor was (OR=8.39; $CI_{95\%}$ 1.91-36.78) in het voorspellen van een correct resultaat. De resultaten gaven aan dat de sensitiviteit voor RS-virus diagnose in Europa goed is en dat real-time PCR bij voorkeur wordt gebruikt voor RS-virus diagnostiek. In **Hoofdstuk 7** wordt de detectie van respiratoire virussen gepresenteerd voor zuigelingen tijdens de eerste respiratoire infectie en is de sensitiviteit van neuswatten and nasopharyngeale aspiraten onderzocht. Tevens is gekeken naar de rol van patiënt eigenschappen en hoeveelheid virus ("virale load") voor de sensitiviteit. Gepaarde nasopharyngeale aspiraten en neuswatten werden afgenomen bij 98 zuigelingen. Rhinovirus (n=67) en RS-virus (n=39) werden het meest frequent gedetecteerd. Meer dan 1 pathogeen werd gedetecteerd in 48% (n=45) van de kinderen. De sensitiviteit van de neuswat was lager dan het nasopharyngeale aspiraat, dit was meest duidelijk voor de detectie van het RS-virus (51% vs. 100%) en rhinovirus (75% vs. 97%). De sensitiviteit van de test voor de afgenomen neuswat was sterk gerelateerd met de Ct waarde (Cycle threshold) ($p<0.001$). Voor epidemiologische studies en surveillance doeleinden kan de neuswat worden gebruikt. Een nadeel is echter dat de sensitiviteit van de test lager is dan bij het aspiraat, dit speelt vooral een rol bij mildere infecties van RS-virus. In **Hoofdstuk 8** ten slotte, worden de resultaten van eerdere hoofdstukken samengevat. RS-virus surveillance kan gebruikt worden ter ondersteuning van klinische besluitvorming voor profylaxe in premature zuigelingen. Tevens helpt RS-virus surveillance bij het beter begrijpen van geografische trends van respiratoire infecties. De bevindingen in dit proefschrift zorgen voor aanknopingspunten om de surveillance verder te ontwikkelen op zowel regionaal als Europees niveau. RS-virus surveillance is relevant voor kennis over "wie" geïnfecteerd is en "wanneer", en geeft nuttige informatie wat de beste tijd is voor het toedienen van palivizumab. Tevens is een volledig geïntegreerd surveillance systeem - van klinische diagnose tot ziekenhuisopname - van groot belang en kan in de toekomst nuttig zijn voor effectiviteitonderzoek van een vaccin tegen het RS-virus.

Dankwoord

Dankwoord

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About the author

About the author

Tamara Janicke Meerhoff was born on 21 September 1977 in Zaanstad, the Netherlands. She followed her secondary education in Zaandam (Pascal College) from which she graduated in 1996. She studied Medical Biology at the VU University Amsterdam from which she graduated in 2000. As of April 2001, she started working as a researcher at NIVEL (Netherlands Institute of Public Health and Research). From 2001 to 2004 she worked on the co-ordination of influenza surveillance in Europe. As of 2004 she started to work on a PhD on RSV in addition to the work on influenza surveillance. During the work at NIVEL she completed the Master program Epidemiology at the VU Medical Centre in Amsterdam, from which she graduated in 2007. Tamara is married to Pedro Nooijen en together they have two children: Arvid (2003) and Tibor (2006).