Impact of molecular diagnostics for the detection of respiratory viruses and clinical value

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

(Herbert AP. The common cold. In : Look back and laugh.London:Methuen, 1960: 115-117.)

Voor Jaap Jan en Merle

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Chapter 1

General Introduction

Acute respiratory tract infections (RTI's) affect the airways and lungs and can be divided into upper RTI's (URTI), including the common cold, sinusitis and pharyngitis, and lower RTI's (LRTI), including bronchitis and pneumonia.

The viruses and bacteria that are responsible for URTI are listed in table 1. Viruses are the most common invaders of the most upper part of the upper respiratory tract, the nasopharynx, and a large variety of types is responsible for the common cold [29]. Bacteria account for the remainder of pathogens and are mostly involved in the lower part of the upper respiratory tract, causing acute pharyngitis and sinusitis.

Table 1. Most common causative pathogens in URTI

Viruses	Bacteria
Rhinoviruses	*Strep. pneumoniae*
Coronaviruses	*Strep. pyogenes*
Parainfluenza viruses	H. influenzae
Influenza viruses	*Staph. aureus*
Respiratory syncytial viruses	*Neisseria gonorrhoeae*
Coxsackie virus A	*Corynebacterium diphtheriae*
Adenoviruses	
Epstein-Barr virus	
Herpes simplex virus type 1	

Acute infections involving the lower respiratory tract are usually more severe than the URTI's and include bronchitis, bronchiolitis and pneumonia. Some of the same viruses and bacteria that are responsible for the URTI's are implicated in the LRTI's as well. A wide range of micro-organisms can cause LRTI's and the etiology varies with age, underlying disorders and exposure to pathogens through occupation, travel or contact with animals. The most severe presentation of LRTI is usually pneumonia, and consequently many studies have focused on the etiology and epidemiology of either community or hospital acquired pneumonia.

The most common pathogens found in pneumonia are shown in table 2. Clinically, viral pneumonias are difficult to differentiate from bacterial pneumonias. Therefore, the incidence of viral pneumonias is almost certainly underestimated. In general, bacteria of which *S. pneumoniae* is the most common pathogen, account for approximately 50-60% of community acquired pneumonia, 10-18% of episodes of pneumonia are attributed to viruses, and the rest are either ascribed to fungi or remain unknown [5,54]. The majority of cases of nosocomial pneumonia (= 72 hours after hospitalization) are

caused by Gram negative bacilli and *S.aureus.* Rarer etiologic agents include *Streptococcus pneumoniae*, anaerobes, influenza A, Legionella species, and fungal pathogens [73]. The role viruses play in hospital acquired pneumonias is not well documented except for outbreaks of influenza, parainfluenza and respiratory syncytial virus (RSV) infections [24,36,60,72].

Table 2. Most common causative pathogens in pneumonia

Community-Acquired			Hospital-Acquired
Bacteria	*Viruses*	*Fungi*	
Mycoplasma pneumoniae	Influenza viruses	*Histoplasma* spp.	Enteric aerobic gram-negative bacilli*
Streptococcus pneumoniae	Cytomegalovirus	*Coccidioides* spp	Pseudomonas aeruginosa*
Haemophilus influenzae	Respiratory syncytial viruses	*Blastomyces* spp.	Staphylococcus aureus*
Chlamydia pneumoniae	Measles virus		Oral anaerobes*
Legionella pneumophila	Varicella zoster virus		
Moraxella catarrhalis	Hanta virus		
Mycobacterium tuberculosis			
Chlamydia psittaci			
Bordetella pertussis			

* Bacteria

Respiratory viruses as a cause of acute respiratory tract infection

The variety and omnipresence of respiratory viruses along with their ease to spread among human populations ensure their occurrence as causes of infection and illness in all human populations. This includes persons of all ages, the healthy and the unhealthy, and the immunocompetent and the immunocompromised. Viral respiratory infections are associated with considerable costs in terms of decreased productivity and time lost from work, visits to health-care providers, and the amount of drugs prescribed [7,62,63] . Respiratory viruses are known to cause substantial morbidity in elderly people living in the community [49]. More over, increasing evidence exists that respiratory virus infections trigger serious acute respiratory conditions that result in hospitalization of patients with underlying condition [12,19,30]. Large epidemiologic studies that have been conducted in the 60's and 70's give a good insight on the incidence of acute respiratory infection in the community [45-47]. The annual frequency of these respiratory illnesses rapidly falls after the second year of life and increases again in frequency at age 20 to 29 years, probably by exposure of family members to young children

with respiratory symptoms [47]. These and other community based studies have been pivotal in establishing the present views on the cause of common viral respiratory infections. Depending on the diagnostic techniques that have been used and the population that was studied viral pathogens have been reported to be the cause of acute respiratory illness in the community in between 25% to 69% of cases [44,46,49]. Of the respiratory viruses, rhinoviruses have consistently been identified as the most frequent causative pathogen, accounting for up to 52% of the infections (table 3) [46]. The relative proportion of the other different respiratory viruses vary largely, depending on factors such as age, season and diagnostic techniques.

Table 3. Respiratory viruses in the common cold

Virus	Virus family	Estimated proportion in common cold
Rhinoviruses	Picornaviridae	30-50%
Coronaviruses	*Coronaviridae*	10-15%
Influenza viruses	*Orthomyxoviridae*	5-15%
Parainfluenza viruses	*Paramyxoviridae*	5%
Respiratory syncytial virus	*Paramyxoviridae*	5%
Enteroviruses	*Picornaviridae*	<5%
Adenoviruses	*Adenoviridae*	<5%
Metapneumovirus	*Paramyxoviridae*	Unknown

As said, respiratory viruses are usually associated with URTI's, mainly the common cold, and the respiratory viruses that are responsible for the majority of acute respiratory illnesses and their prevalence in the common cold are shown in table 3. Despite the availability of sophisticated diagnostic methods, about 20-30% remain without a proven viral cause [42].

It is difficult to distinguish the respiratory viruses that cause acute respiratory tract infection on clinical grounds only and although often associated with URTI, including the common cold, their clinical presentation may range from asymptomatic to severe illness. Most of these respiratory viruses belong to the group of RNA stranded viruses and each of the viruses also has its specific clinical entity. Rhinoviruses (HRV) are members of the *Picornaviridae* family and were first discovered in 1956. Since then, more than 100 different serotypes have been identified. Human coronaviruses (HCV), members of the *Coronaviridae* family, were first identified in 1962. The human strains are

divided into two distinct antigenic groups which are both represented by a prototype virus, HCV 229E and HCV OC43. Rhinoviruses and coronaviruses are the most frequently identified causes of the "common cold" syndrome [3,42,46].

RSV, parainfluenza viruses and the recently discovered human metapneumovirus (hMPV) are all members of the *Paramyxoviridae* family.

For human RSV, two antigenic subtypes, A and B, are distinguished. RSV is mainly associated with viral respiratory tract disease in young children and infants. It is the most common cause of bronchiolitis in infants, with up to 10% of those infected requiring specialized pediatric care [51,61].

The four serotypes of human parainfluenza viruses (hPIVs) belong to the *Paramyxovirus* (hPIV type 1(hPIV-1) and hPIV-3) or *Rubulavirus* (hPIV-2 and hPIV-4) genera of the *Paramyxoviridae* family. The major clinical manifestations of infection with hPIV-1,-2, and –3 are croup, pharyngitis, and upper respiratory infections in children but they are also well recognized as causes of bronchiolitis, and pneumonia in infants and very young children [39]. The newly discovered hMPV appears to be similar to the disease caused by human RSV, and has been associated with severe ARI in young children, elderly people and immunocompromised children [6,17,21,38,66].

Influenza viruses constitute the only genus of *Orthomyxoviridae* family. There are three types of influenza viruses, namely A, B, and C which are distinguished by serological responses to their internal proteins. Influenza virus A and B cause the most prominent infection in humans. Influenza viruses are unique among respiratory viruses in their capacity to cause regularly yearly epidemics that are associated with excess morbidity and mortality, manifested as excess rates of pneumonia and influenza associated hospitalization during influenza epidemics [57,64].

The human adenoviruses are nonenveloped, double-stranded DNA viruses. They belong to the family *Adenoviridae* and have been classified into six subgenera, A to F. The subgenera B, C, and E have been associated with respiratory disease. Double-stranded DNA viruses have the ability to persist in airway epithelial cells long after the acute infection has cleared. The spectrum of adenovirus infection can range from asymptomatic shedding to fatal disseminated disease in patients who have undergone organ or bone marrow transplantation. Disease can be caused by primary infection, reactivation of latent infection in the transplant recipient, or reactivation of infection transmitted in the donated organ [10,65].

Although these viruses are usually associated with the common cold and an uncomplicated and benign course of disease, awareness is growing that these virus infections can have serious consequences.

Respiratory viruses and underlying disease.

Respiratory viruses that are identified as major pathogens that present with the common cold are becoming more and more acknowledged as a cause or contributor to severe disease in particular patient groups. Among these groups are the immunocompromised patients in whom respiratory

viruses are a contributor to severe disease and death and patients with underlying lung disease (f.e. asthma and chronic obstructive pulmonary disease) in whom they are a major cause of acute respiratory insufficiency. These two groups will be further addressed here.

Over the past decades, there has been growing recognition that the regular community respiratory viruses, which are highly prevalent among immunocompetent persons with respiratory illness, are also highly prevalent among adult immunocompromised cancer patients experiencing a respiratory illness [32,41,68,70]. Prospective surveillance studies in the United States showed that community acquired respiratory virus infections were diagnosed in approximately 31% of the adult bone marrow transplant recipients that were hospitalized with acute respiratory illness [70]. In contrast to the usually benign, self-limiting course of respiratory virus infection in the healthy adult, high frequencies of pneumonia and death were associated with respiratory virus infection in the immunocompromised patient with cancer [14,20,43,53,68,70]. Overall frequencies of pneumonia and death appear similar for each virus, but RSV infection has been associated with an exceptionally high frequency of fatal pneumonia in bone marrow transplant recipients or in patients receiving chemotherapy [24,69]. Although studied in several patient groups, the role of respiratory virus infections as the cause of severe pulmonary complication in patients receiving chemotherapy or undergoing stem-cell transplantation is not yet sufficiently clarified and may be underestimated in previous studies, particularly in those studies that relied on virus culture.
The clinical association between acute viral respiratory tract infections and increased symptoms of asthma have been well recognized, but the potential importance of the association was probably underestimated until relatively recently when it became clear that up to 85% of asthma attacks in children and an estimated 44% in adults are precipitated by upper respiratory infections [35,50]. Recent studies have focused on the question whether virus infections exacerbate asthma directly by local mechanisms following lower airway infection, or whether they infect only the upper respiratory tract and affect the lower airway indirectly.

Treatment and prevention of acute respiratory virus infection

In general the control of viral respiratory illness is achieved by i) prevention of exposure ii) provision of immunity, and iii) administration of antivirals.
At present, specific antiviral treatments for respiratory viruses are available only for influenza viruses. The use of amantadine and rimantadine is limited by their side effects, their inefficacy against influenza B viruses, and the rapid development of resistant viral strains during treatment [9,58]. The new influenzavirus-specific antivirals, zanamivir and oseltamivir, have fewer side-effects, and are effective against both influenza A and B when administred within 48 hours of the onset of symptoms

[25,27]. Scarce evidence exists for the efficacy of these drugs in the prevention of complications, especially in high-risk patients [1].

There is no specific treatment for RSV infections besides supportive treatment. Ribavirin, an agent with a broad spectrum of antiviral efficacy, is approved in aerosol for treatment of severe RSV infection in infants although controversy exists concerning the overall benefits of ribavirin aerosol therapy [23,67].

Recent advances in antirhinoviral drugs include the development of pleconaril and ruprintrivir. Pleconaril, a novel viral capsid binder, is administered orally and is active against a wide range of rhinoviruses and enteroviruses [26,37,52]. Ruprintrivir, a human rhinovirus 3C protease inhibitor, has demonstrated potent antiviral activity in vitro against a variety of different human rhinovirus serotypes, results in vivo have not been published up to now [33].

Laboratory diagnosis of acute respiratory virus infection

Laboratory conformation of respiratory virus infection is essential to i) determine the cause of illness and thereby gain insight in the prognosis, ii) apply treatment, either supportive or with antiviral agents, iii) to implement preventive measures and iv) obtain information on the pathogenesis of the infection.

Methods for the diagnosis of respiratory virus infections include serology, virus culture, antigen detection, and polymerase chain reaction (PCR). Virus isolation via cell culture, shell vial culture, and antigen detection are the methods most often used for the laboratory diagnosis of respiratory virus infection.

Serology has classically been considered as the gold standard. The major disadvantage of a diagnosis based on the detection of antibodies in a patient's serum is that it is retrospective and has no role in the early, rapid diagnosis as 2-4 weeks must elapse before a significant rise of IgG antibodies produced in response to the infection are detectable. Moreover, it cannot be used for the detection of viruses with a large variety of subtypes circulating at the same time.

At present, viral culture is considered as the gold standard for the laboratory diagnosis of respiratory virus infections. Virus isolation through cell culture is essential to obtain virus isolates for antigenic characterization. Culture has often little value for clinical practice since it may take up to 10 days before results become available. Also the sensitivity is limited since the concentrations of viable virus can decline rapidly in patients after the first days of the infection and the virus can become undetectable by culture in the later course of infection [40]. Moreover, it is labor-intensive and depends on optimal sample transport for sensitive virus isolation.

Alternative diagnostic techniques, such as viral antigen detection (immunofluorescence and enzyme immunoassay techniques) and centrifuge-enhanced shell vial cultures (SVC) combined with immunofluorescent (IF) staining have been introduced in the routine laboratory setting. These techniques provide more rapid results, but are considered to be less sensitive and specific than

conventional cell culture, especially in adults and the elder patients in whom the presence of infected cells in clinical specimens is generally low [16,49,56]. Also, these techniques cannot be routinely used for the detection of all respiratory viruses; for example, antigen testing for rhinoviruses is not possible, because of the variety of different subtypes that exist and co-circulate at the same time[55].

As the respiratory viruses become more acknowledged as important pathogens and with the development of new treatment options, there is increasing interest in the development of rapid laboratory diagnostic methods. To overcome the lack of sensitivity of antigen testing and also to obtain rapid diagnostic results, various PCR assays, either in single or multiplex format, have been developed for the specific detection and subtyping of respiratory viruses [2,4,11,13,22,48,59,71]. PCR has been proven to be very sensitive and specific, but unfortunately is often difficult to implement in a routine diagnostic setting as it still requires time-consuming sample handling and post-PCR analysis [4,8,15,34]. The recent introduction of kinetic or "real-time" PCR allows the detection of amplified DNA as the amplification process progresses. [18,28,31]. The monitoring of accumulating amplicon in real time has been made possible by the labeling of the probe with fluorogenic molecules. This method provides a lot of advantages compared to the former PCR methods by the increased speed, the removal of post-PCR detection procedures, the use of fluorogenic labels and sensitive methods of detecting their emissions, the possibility to quantify the viral load and the use of a uniform protocol.

Aims and outline of this thesis

The scope for this thesis was to improve rapid and reliable detection of respiratory virus infections (chapters 2-6). To be able to allow a more adequate patient care strategy we aimed to i) develop molecular diagnostic techniques feasible for use in a routine laboratory setting (chapters 4-6), and to evaluate their clinical impact , and ii) to use these techniques to clarify the role of respiratory viruses in specific patient groups at risk (chapters 7-9).

With the development of new antiviral agents against various respiratory viruses, the interest in respiratory viruses has increased over the last years. Up to now this development has led to two registered antiviral agents for the treatment of influenza A and B virus infection. In Chapter 2 these two antiviral agents are reviewed.

General practitioners usually do not have direct access to reliable specialized laboratory detection techniques. Since they see most of the patients who might benefit from antiviral therapy, we have tried to define clinical parameters that can be used by the general practitioner to distinguish between infection by influenza viruses and by other viruses (Chapter 3).

The past decade has seen tremendous developments in molecular diagnostic techniques. Chapters 4-6 describe the development of real-time RT-PCR techniques for the detection of influenza A and B viruses, RSV and human coronaviruses OC43 and 229E. The described techniques can easily be

implemented in a routine diagnostic setting. They do not require time-consuming sample handling and post-PCR analysis is automated, excluding serious hazards for amplification product carryover.
In Chapter 4 a real-time Taqman based RT-PCR was developed and validated for the simultaneous and quantative detection of influenza virus A and B. Chapter 5 describes the development of a similar assay for the detection of RSV and the clinical value of this particular assay in immunocompromised patients. The detection of human coronaviruses has always been hampered by the fastidiousness to grow these viruses in cell culture. In Chapter 6 the real-time RT-PCR technique is used for the development of a sensitive method for the detection of human coronaviruses OC43 and 229E.
These, or similar molecular diagnostic techniques form the basis for the detection of respiratory viruses in the studies further described in this thesis.
Chapters 7 and 8 focus on the occurence of respiratory viruses in immunocompromised adults.
A retrospective study using the previously developed molecular techniques was performed to determine the role of respiratory viruses in hematological cancer patients with pneumonia (Chapter 7). Chapter 8 describes the use of molecular diagnostic techniques for screening for respiratory viruses in a prospective surveillance in patients who underwent a bone marrow transplantation.
Clinical observations have suggested that patients with allergic rhinitis and asthma experience more pronounced symptoms during a viral upper respiratory tract infection (URI) than patients who do not have allergies and who are infected with the same virus under similar circumstances. In Chapter 9 we have studied the role of respiratory viruses, in particular rhinoviruses and influenza viruses, in asthmatic and non asthmatic subjects during URTI and tried to address the question whether it is the host response to the virus or the virus itself that plays the major role in the development of symptoms.
Finally, in Chapter 10 the work of the preceding chapters is summarized and discussed.

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Chapter 2

New antiviral agents for the prevention and treatment of influenza

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ABSTRACT

Influenza is an important epidemic viral infection and a cause of excess morbidity and mortality. Immunization with inactivated vaccines remains the main strategy to prevent infection with the influenza virus and to reduce complications. A new class of antiviral agents, the neuraminidase inhibitors, was recently developed for the prevention and treatment of influenza. Neuraminidase is one of two glycoproteins on the surface of the influenza virus and has enzymatic activity. Inhibition of its activity prevents the spread of new viral particles. Several clinical trials report that treatment with the neuraminidase inhibitors zanamivir and oseltamivir effectively reduces the symptom score and the duration of symptoms. These drugs are also effective in preventing infection with influenza virus A and B. Although resistance to neuraminidase inhibitors appears to be limited, surveillance for the emergence of resistant variants is important. The neuraminidase inhibitors may prove to be a useful addition to yearly vaccination.

INTRODUCTION

Influenza is an important cause of disease and death worldwide, especially among elderly individuals and patients at risk, such as those with diabetes mellitus, chronic heart disease, or chronic obstructive pulmonary disease (COPD). The disease course is complicated in such patients, and morbidity and mortality are high. An estimated 1.5 million Dutch people contract influenza a year, 200,000 to 400,000 of whom consult their general physician; of these patients, 10,000 to 20,000 are hospitalized. About 2000 deaths occur annually due to the direct or indirect consequences of influenza [6,19,27]. The annual influenza epidemic also has substantial economic consequences, in terms of absence from school and work [26]. In the United States, direct costs are estimated at US$ 1 to 3 billion annually, while indirect costs are even higher, reaching US$10–15 billion annually [21].

Vaccination with an inactivated influenza vaccine is currently the most effective way to prevent influenza and to limit the symptoms and complications of infection. The Health Council annually presents its advice concerning vaccination policy. Since the introduction of a general practice-based influenza vaccination program in the Netherlands, the number of patients annually vaccinated has increased substantially: all high-risk patients, about 20% of the population, are called up for vaccination, and 77% of such patients are actually vaccinated [11,29]. However, influenza cannot be completely prevented by vaccination. For instance, the protective effect of vaccination is limited in some patients with a reduced immunocompetence. Furthermore, antigenic drift of influenza viruses makes it difficult to predict what the composition of the vaccine should be. Every 10 to 20 years the influenza virus changes to such an extent that a completely new subtype develops (antigenic shift). A

well-matched vaccine offers effective protection to 70–90% of healthy young adults and to 30–50% of people aged 65 years and older [3,9].

Existing antiviral drugs for the influenza viruses, amantidine and rimantidine, have been shown to be active both therapeutically and prophylactically. These drugs provide prophylactic protection in 70–90% of healthy young adults [7]. Despite this, these drugs are used scarcely. Only amantadine is approved in the Netherlands, but it is seldom prescribed. This restricted use of amantadine and rimantadine is probably due to their side effects: (i) they are not active against influenza virus B; (ii) they give rise rapidly to reduced susceptible strains; and (iii) amantadine in particular has severe side effects [5,28]. In addition, influenza is often unjustly considered a relatively harmless infectious disease and treatment as not being necessary. A new class of antiviral drugs with a different mechanism of action has recently been developed to prevent (prophylactic use) or to treat (therapeutic use) influenza A and B infections. Various clinical studies have been performed with two such agents, zanamivir (Relenza®, GG167) and oseltamivir (Tamiflu®, GS4104, an ethyl ester whose active metabolite is GS4071). Zanamivir is approved for therapeutic use in Europe, the United States, and Australia, whereas oseltamivir is approved for therapeutic and prophylactic use in only Switzerland and the United States. The question is what place these new drugs will have in the treatment of a patient with influenza and in the prevention of influenza (as adjunct to the annual vaccination). In this article, the mechanisms of action, the results of clinical trials, and the possible applications of these new drugs are discussed.

NEURAMINIDASE INHIBITORS

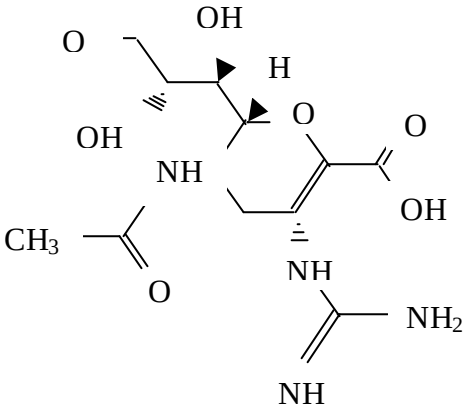
Both zanamivir and oseltamivir inhibit viral neuraminidase. It is one of the two cell-surface proteins on the influenza virus and has enzymatic activity. Neuraminidase plays an essential role in virus replication, by cleaving the bond that attaches new virus particles to the surface of an infected cell. The release of new virus particles facilitates the spread of virus in the airways and promotes virus penetration of the mucous lining of the respiratory tract [4].

Neuraminidase inhibitors were designed by means of computer modeling [31]. The inhibitors bear a strong structural resemblance to the natural substrate of neuraminidase, sialic acid (Figure 1). By binding to the active center of neuraminidase, the inhibitors block enzyme activity. Thus neuraminidase inhibitors indirectly inhibit virus replication; their direct action is to prevent the dissemination of newly formed virus particles. However, the symptoms of influenza are caused not only by virus replication but also by the immunological defense. Neuraminidase inhibitors do not have a direct effect on the systemic immunological response to virus infection. In order to inhibit virus replication, neuraminidase inhibitors should be administered early, within 48 hours, but preferably within 24 hours, of the onset of symptoms of influenza.

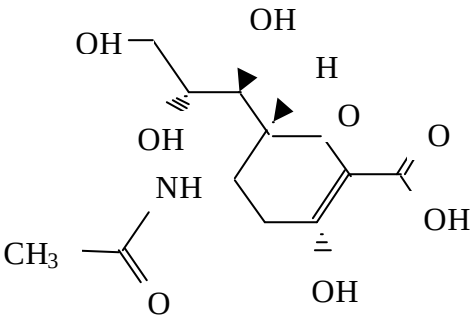
ZANAMIVIR

Zanamivir (Figure 1) actively inhibits the replication of influenza virus A and B both *in vivo* and *in vitro*, even in isolates that are less sensitive to amantadine and rimantadine [16,33]. Its bioavailability is low after oral administration, in part because zanamivir is rapidly cleared by the kidneys. For this reason, local therapy is preferred, with administration by inhalation or intranasal application.

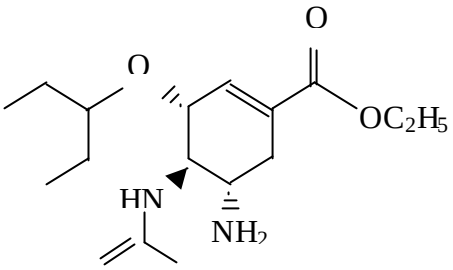
Figure 1. Structure of neuraminidase inhibitors and the natural substrate



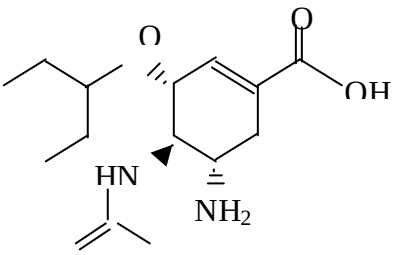
Zanamivir (GG167)



Natural substrate
(sialic acid)



Oseltamivir (GS4104)



Active metabolite (GS4071)

Therapeutic action (table 1). Phase II research into dosage forms, safety, and therapeutic effect has shown that zanamivir, administered intranasally or by inhalation, is effective against influenza virus A and B [14,16]. The MIST (Management of Influenza in the Southern hemisphere Trialist) multicenter phase III study of the therapeutic effect of zanamivir showed that a 5-day treatment with zanamivir (10 mg, twice daily, by inhalator), administered within 36 hours of the onset of flu symptoms, diminished the duration of symptoms by 1.5 days compared with placebo [1]. In a small group of patients at risk (n = 76), consisting mainly of patients with mild asthma, the duration of symptoms was reduced by 2.5 days.

Table 1. Therapy with zanamivir or oseltamivir

Study	Dose	Symptom duration*	Virus excretion (AUC)
Zanamivir			
Hayden et al.[16] Phase II (*n*=95)	2-6 dd 16 mg 4 days	40-60% decrease in symptoms	87%
Hayden et al.[14] Phase III (*n*=417)	2 dd 6.4 mg IN + 10 mg IH 2 dd 10 mg IH 5 days	1-3 days 1-3 days	Significant -
MIST Study Group[1] Phase III (*n*=455)	2 dd 10 mg IH 5 days	1.5-2.5 days	NM
Oseltamivir			
Hayden et al.[15] Phase II (*n*=69)	2 dd 20 mg p.o. 2 dd 100 mg p.o. 1-2 dd 200 mg p.o.	42 hours	Significant
Hayden et al.[17] Phase II (*n*=117)	2 dd 75 mg p.o.	No symptoms**	83%
Nicholson et al.[23] Phase III (*n*=726)	2 dd 75-150 mg p.o.	29-35 hours 43-47 hours***	30-40%

IN= intranasal, IH= inhaled, NM, not measured, AUC= area under the curve.
* Compared with placebo; ** infection with influenza virus B; *** administration within 24 hours

There were also significantly fewer complications in this group, and the prescription of antibiotics was lower than in the placebo group. Possible side effects, such as headache and nausea, occurred to the same extent in the placebo and treatment groups. A phase III study carried out in Europe and the United States showed that zanamivir reduced the duration of illness by 3 days in comparison with placebo, provided that it was administered within 30 hours of the first flu-like symptoms [14]. In the Netherlands, zanamivir (sold under the name Relenza®) is licensed for therapeutic indications in patients older than 12 years. The treatment regimen is 10 mg per inhalator (Diskhaler®), twice daily for 5 days.

Prophylactic action (table 2). Research with experimentally infected volunteers showed that zanamivir, given prophylactically, could prevent a laboratory verified infection with influenza virus in 82% of the cases. Signs of influenza were diminished by 50–80% in those volunteers who became infected [16]. In a randomized, double-blind, placebo-controlled phase III study of the prophylactic use of zanamivir (10 mg, once daily for 4 weeks), the drug

was shown to be 67% effective compared with placebo in preventing infection with influenza virus and 84% effective in preventing infection with influenza virus accompanied by fever. Side effects were comparable in the drug- and placebo-treated groups [22]. In a research setting, zanamivir has been shown to protect against influenza virus infection when used prophylactically within a household in which one member is infected with the influenza virus [13].

Table 2. Profylactic studies with zanamivir and oseltamvir

Study	Dose/duration	Efficacy compared to placebo*
Zanamivir		
Hayden et al.[16] Phase II (*n*=104)	2-6 dd 16 mg IN 2 dd 3.6 mg IN/IH 2 dd 7.2 mg IH	82%/ 95%
Calfee et al.[2] Phase II (*n*=16)	2 dd 600 mg i.v. 5 days	86%/ 100%
Monto et al.[22] Phase III (*n*=1107)	1 dd 10 mg IH 4 weeks	67%/ 84%
Oseltamivir		
Hayden et al.[17] Phase II (*n*=33)	1-2 dd 100 mg p.o.	61%/ 100%
Hayden et al.[12] Phase III (*n*=1559)	1-2 dd 75 mg p.o. 6 weeks	74%/ 82%

IN= intranasal, IH= inhaled, i.v.= intravenous
* Laboratory confirmed influenza without/with fever

A small-scale study in a nursing home showed that zanamivir provides protection against an influenza epidemic in a closed community [25].

Antiviral resistance. A mutant influenza virus with reduced sensitivity to zanamivir has been detected only once in clinical trials with the drug. It was isolated from a child with diminished resistance who was infected with influenza virus B [10].

OSELTAMIVIR

Oseltamivir is a new neuraminidase inhibitor with excellent oral bioavailability. After oral administration, the precursor is converted into the active metabolite (Figure 1). The *in vitro* efficacy of oseltamivir against neuraminidase and virus replication is similar to that of

zanamivir [15]. In addition to subtypes of the influenza virus known to be active in humans (H1N1, H2N2, H3N2), two new pathogenic influenza variants (H5N1, H9N2) have proved susceptible to oseltamivir [20].

Therapeutic action (table 1). The effective dose (20, 100, 200 mg twice daily, or 200 mg once daily), therapeutic and prophylactic effect, and safety of oseltamivir were investigated in a placebo-controlled, double-blind study involving experimentally infected volunteers. The duration of symptoms was shortened by about 42 hours compared with placebo, irrespective of the dose used. The inflammatory response was monitored by measuring a number of important proinflammatory cytokines, namely, interleukin 6 (IL-6), tumor necrosis factor a (TNF-a), and interferon-? (IFN-?). The concentrations of these proinflammatory cytokines were significantly lower in oseltamivir-treated volunteers than in placebo-treated volunteers. Mild side effects, such as headache and nausea, occurred significantly more often in the oseltamivir group (18%) than in the placebo group (7%); however, these side effects did not constitute a reason to prematurely stop treatment [17]. In a phase III clinical trial, oseltamivir (75–100 mg, twice daily for 5 days) reduced the duration of illness by 1.5 days [23,30].

Prophylactic action (table 2). Prophylactic oseltamivir provided 100% protection against experimental infection of volunteers in a phase II study [17]. Indeed, prophylactic oseltamivir (75 mg, once or twice daily for 6 weeks) was found to be tolerated well and to provide 74% protection compared with placebo in a clinical study by Hayden et al [12]. However, nausea (12.1% versus 7.1%) and headache (2.5% versus 0.8%) occurred more often in the oseltamivir-treated subjects than in the placebo-treated subjects. A number of studies have investigated the prophylactic action of oseltamivir in a closed community. In a double-blind, placebo-controlled, phase III study of the prophylactic efficacy of oseltamivir in people older than 65 years, the drug was shown to have an efficacy of 92% compared with placebo. During a flu epidemic, oseltamivir (75 mg, once daily) or placebo was given for 6 weeks; 438 of the 548 (80%) of the subjects were also vaccinated. Oseltamivir was tolerated well [24]. The efficacy of a short prophylactic treatment (5 days) with oseltamivir was investigated in the relatives of patients infected with the influenza virus. Compared with placebo, the efficacy of oseltamivir in preventing virus transmission was 89% [32].

Antiviral resistance. The development of resistant strains of the influenza viruses to oseltamivir was investigated during clinical studies. Four influenza virus strains with a diminished sensitivity to oseltamivir were isolated from 418 treated patients [18].

DISCUSSION

Annual epidemics of influenza have a substantial impact on public health and on health care facilities. Moreover, there is a real threat of a new pandemic. As long as vaccination fails to provide total protection, there is an urgent need for other strategies to prevent or treat

influenza. In addition to the development of newer vaccines, the development of antiviral drugs is an interesting option. Research published to date shows that zanamivir and oseltamivir are able to slow the replication of influenza virus A and influenza virus B, both *in vitro* and *in vivo*, [16,25] and to limit the severity of the disease. Severe side effects have not been reported [1,14,17]. These are promising results that need to be substantiated in further clinical studies.

Although vaccination remains an important strategy to prevent influenza and to reduce complications, there are a number of possible applications of neuraminidase inhibitors as adjunct to vaccination (table 3).

Treatment. Timely treatment with zanamivir reduces the duration of illness by a mean of 1.5 days in an otherwise healthy population, and by 2.5 days in patients at risk [1]. In addition to a reduced disease burden, this can be economically advantageous in an otherwise healthy population. Neuraminidase inhibitors would appear to be indicated for patients from risk groups if these are not vaccinated or if vaccination can be expected to provide insufficient protection. It has not yet been convincingly demonstrated that these drugs reduce complications. Although there is a trend to reduced antibiotic use and fewer complications, the groups investigated were too small to enable valid conclusions to be drawn [1]. The therapeutic use of neuraminidase inhibitors is, however, complicated [8]. Both inhibitors must be administered in an early phase to be effective, preferably within 30 hours of symptom onset. However, at present there is no quick, sensitive, and reliable test to establish the diagnosis available to first-line healthcare professionals.

Table 3. Possible uses of neuraminidase inhibitors during a flu epidemic

Therapy:*
(administration < 48 hours after onset of first symptoms)
1. Patients at risk (especially when there is vaccine "mismatch")
2. During pandemic (until vaccine becomes available): both healthy and at-risk patients
3. (Ultimately healthy, non-vaccinated individuals)

Prophylaxis:*
1. Non-vaccinated at-risk patients (vaccinate at the same time!)
2. At-risk patients in closed communities when there is vaccine "mismatch" (nursing homes)
3. During a pandemic (until vaccine becomes available): at-risk patients

* > 12 years

A standardized questionnaire, additional to the existing Nederlands Huisartsen Genootschap (Dutch College of General practitioners) standard on influenza and vaccination against

influenza, might be useful to identify possibly infected patients during an influenza epidemic. Moreover, patients often go to their general physician in a late phase.

Prophylaxis. Prophylactic use may be appropriate if the vaccine is not adequately matched with the circulating strains or when there is a pandemic. The new antiviral agents can provide protection while a new vaccine is awaited and during the first 2 to 3 weeks after vaccination. Prophylactic administration would certainly be a worthwhile adjunct to existing treatment possibilities in at-risk patients in closed communities, such as nursing homes or hospitals, when vaccination provides inadequate protection, or when there is a risk of influenza spreading in a hospital. However, there are disadvantages to the prophylactic use of these drugs. For example, resistance may develop if the drugs are administered long term, although at the moment this seems to be less of a problem with neuraminidase inhibitors than with amantadine and rimantadine. The binding site of the neuraminidase inhibitors is well conserved, and the inhibitors bear a strong resemblance to the natural substrate of neuraminidase. However, strains with a reduced sensitivity to zanamivir and oseltamivir have been detected in clinical studies [13,18]. Thus care must be taken to monitor influenza viruses for the emergence of resistant strains or strains with diminished sensitivity to neuraminidase inhibitors. A second disadvantage is the cost of prophylactic administration. Lastly, it is possible that long-term use gives rise to adverse effects.

Zanamivir and oseltamivir have a similar efficacy and only differ in their dosage form. Zanamivir is inhaled, a route of administration that may cause problems in young children or frail elderly patients. A potential advantage of inhalation is that there is limited systemic exposure. In contrast, oseltamivir undergoes renal clearance and gastrointestinal side effects have been reported [2]. Vaccination remains the most important and effective strategy to prevent influenza and its complications. However, as it is not yet possible to provide total protection by vaccination, new possibilities to prevent and treat this infectious disease remain welcome.

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Chapter 3

Clinical diagnosis of influenza virus infection: evaluation of diagnostic tools in general practice

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ABSTRACT

Background. With the development of new antiviral agents for influenza, the urge for rapid and reliable diagnosis of influenza becomes increasingly important. Respiratory virus infections are difficult to distinguish on clinical grounds. General practitioners however, still depend on their clinical judgement.

Aim. To evaluate the value of clinical symptoms to diagnose influenza virus infection.

Method. A multicentre study with 81 patients from 14 GP's. Patients with fever and at least one constitutional symptom and one respiratory symptom were included. A questionnaire with the medical history and clinical symptoms was completed and a combined nose-throat swab was taken. Virus culture, rapid culture and PCR were performed on each specimen. Multivariate analysis was used to obtain the best predictive model.

Results. By using PCR an increase was seen in the detection of the viral pathogens compared with the results of culture. In 42 of 81 patients PCR was positive for influenza. A positive predictive value (PPV) of 75% was observed for the combination of headache at onset, feverishness at onset, cough, and vaccination status during the period of increase influenza activity. Criteria used by the ICHPPC-2 resulted in a PPV of 54%. The PPV for diagnosis made by the GP was 76%.

Conclusion. Although influenza is difficult to diagnose on clinical grounds, the general practitioners in this study were able to diagnose influenza as such more accurately on their jugment than by the other criteria.

INTRODUCTION

Each year the general practitioner (GP) is confronted with the seasonal local and/or regional influenza epidemic. Although the impact and complications of influenza virus infection are well known [17], active policy by the GPs is limited by the yearly vaccination of people at risk. The problems that are encountered by the GP are the difficulty in distinguishing influenza virus infection clinically from other respiratory infections, the lack of rapid laboratory diagnostic tools and the limited possibilities for intervention.

Recently, promising results have been published of trials with new antiviral compounds, the neuraminidase inhibitors, which are effective against influenza A and B [1,11-13]. They are to be taken within 48 hours of infection to be effective. Two of these agents, zanamivir and oseltamivir, have recently been registered in some countries for treatment. With the development of these new treatment options, rapid diagnosis gains relevance for GPs. In the absence of laboratory tests that are feasible, reliable and rapid, influenza diagnosis still has to be made by evaluation of signs and symptoms.

In this study, we evaluate the value of clinical symptoms to diagnose influenza virus infection for GPs. Most studies so far have focused on hospitalised children or the elderly, either emphasising severe

symptoms or lack of symptoms [10,18,20,22]. Although it is difficult to identify influenza based on clinical characteristics, diagnostic criteria have been formulated. The criteria to differentiate between influenza virus infection and infection caused by other respiratory viruses are not uniform. Guidelines for the diagnosis of influenza are formulated for GP's through the criteria of the International Classification of Health Problems in Primary Care (ICHPPC-2). Influenza is diagnosed when there is an influenza outbreak and a patient has 4 of the following symptoms: sudden onset, contact with influenza, fever, cough, chills, malaise, myalgia, hyperaemic mucous membranes of the nose and throat, or 6 of these symptoms outside an influenza outbreak [8]. The Netherlands institute of primary health care (NIVEL) is running a registration network of 46 sentinel general practices spread over the country. The NIVEL reports patients with acute respiratory illnesses. They define influenza-like illness (ILI) as abrupt onset (prodromal phase with minor symptoms of less than five days), rectally measured body temperature of at least 38ºC and at least one of the following symptoms: cough, coryza, headache, retrosternal pain or myalgia [2].

The aim of this study was to investigate the relation between signs and symptoms and the presence of influenza virus infection and to assess the accuracy of the clinical diagnosis by GPs in patients with an acute respiratory illness. To evaluate the clinical presentation we have chosen to use the more sensitive PCR besides virus culture and antigen testing. The ICHPPC-2 criteria, the sentinel criteria and the results of our clinical questionnaire were matched with the results of the most sensitive laboratory technique.

METHODS

Study design. From November 1997 to May 1998, 14 GPs in the Utrecht region in the Netherlands included patients who presented at their practice with: fever (≥ 38°C, anamnestic), at least 1 constitutional symptom (malaise, headache, myalgia, chills), and at least 1 respiratory symptom (coryza, sneezing, cough, sore throat, hoarseness). Patients were asked to participate when these symptoms existed for ≤ 48 h. A physical examination was carried out by the GP and a questionnaire was completed. A combined nose and throat swab was taken for the laboratory detection of virus. The questionnaire contained the following items: inclusion criteria, administrative data (initials, date of birth, gender), medical history, medication, smoking habits, influenza-vaccination status, presenting symptoms, contact with other patients with similar symptoms, onset of symptoms, physical examination, therapy and the presumed aetiology of illness by the GP prior to the results of the virological diagnosis.

The NIVEL-criteria for increased influenza activity were used: increased influenza activity means that the threshold of 5/10.000 inhabitants with ILI is exceeded. An influenza epidemic is spoken of when the threshold exceeds 40/10.000 inhabitants with ILI [9].

Virological methods. Nose and throat swabs were obtained for virus isolation and either transported to the laboratory in virus transport-medium directly or stored at 4°C for a maximum of 24 hours at the general practice. Part of the patient material was used for immediate culture of influenza viruses, parainfluenza viruses, picorna viruses, RSV, adenoviruses and herpesviruses. After 2 days of culture, rapid antigen testing was performed by

immunofluorescence with monoclonal antibodies for influenza viruses, parainfluenza viruses, RSV and adenovirus (rapid culture).

The remaining material was frozen and stored at -70°C for later analysis. On the remaining material polymerase chain reactions (PCR) were performed for influenza A and B virus, parainfluenza virus 1, 2 and 3, picornaviruses (rhinovirus and enterovirus), respiratory syncytial virus (RSV) and coronaviruses. Rhinoviruses were identified by Bgl I digestion of the picornavirus RT-PCR amplicons [21]. Viral nucleid acid extraction was performed according to the method of Boom et al.[3]. For all PCR reactions a one-tube reverse transcriptase polymerase chain reaction (RT-PCR) was followed by a nested polymerase chain reaction (nested-PCR). Similar RT-PCR and nested-PCR conditions were used as described by M. Nijhuis et al.[19].

Statistical analyses. Data were analysed by Chi Square or Fisher's exact test. Log regression was used for multivariate analysis, using all relevant patient characteristics and influenza symptoms at onset and at presentation ($P<0.15$ in the univariate analysis) as independent variables and positive PCR result for influenza A or B as a dependent variable. $P<0.05$ was considered significant.

RESULTS

Patientpopulation. A total of 81 patients were included by 14 GPs from 1 November 1997 to 1 May 1998. Thirty-three (41%) of the patients were male. Thirty-three patients were aged <25 years and 43 patients were aged between 25-65 years. Only 5 patients were included above 65 years of age. The majority of patients were otherwise healthy individuals.

Table 1 Various respiratory viruses detected by PCR in NT-swabs of patients presenting with Influenza-like illnesses. Values are number of samples.

Virus	*PCR*
Influenza virus A	42
Influenza virus B	-
Picornavirus	5
Respiratory syncytial virus	2
Coronavirus	3
Parainfluenza virus	-
Adenovirus	ND*
No virus detected	29
Total	81

*Adenoviruses were only diagnosed by culture/rapid culture: cell culture yielded one positive result.

Laboratory findings. All of the 33 culture or rapid culture positive samples were tested positive by PCR. In addition, viral pathogens were identified in another 19 patients using PCR. The NT-swabs that were taken of all 81 patients included during the surveillance period yielded 53 pathogens: 42 influenza A viruses, 5 rhinoviruses, 3 coronaviruses, 2 RSV and 1 adenovirus. No mixed infections were found (Table 1).

Viruses were detected in samples from 26/33 (79%) patients <25 years and 26/43 (60%) patients aged between 25-65 years. One out of 5 patients above 65 years (20%) was found positive by PCR.

Distribution of infection. The rate of detection of viruses was not equally distributed during the 6 months surveillance period: influenza virus A was mainly detected during the end of winter and beginning of spring (February through March 1998). According to the NIVEL surveillance data the influenza season was mild: during weeks 8-14 in 1998 there was increased influenza activity and a maximum of 17/10.000

inhabitants with ILI was seen in week 9 [14]. We found that 42/81(52%) patients were indeed infected with influenza virus.

Table 2 Clinical findings of patients with ILI according to PCR result for influenza. Values are numbers of patients (percentages) and relative risk (RR) with 95% confidence intervals (% CI)

Clinical observation	Influenza virus A (n=40)	unknown (n=39)	RR	95% CI
Respiratory symptoms:				
Nasal congestion	27 (67.5)	23 (59)		
Sneezing	13 (32.5)	12(31)		
Cough	39 (97.5)	30(77)**†	11.7	1.4-97.5
Hoarseness	3 (7.5)	9(23)†	0.3	0.7-1.1
Sore throat	32 (80)	27(69)		
Shortness of breath	6 (15)	11 (28)		
General symptoms:				
Headache	28 (70)	22 (56)		
Feverishness	35 (87.5)	33 (85)		
Myalgia	24 (60)	24 (62)		
Malaise	29 (72.5)	29 (74)		
Symptoms of onset:				
Cough	17 (41)	18 (46)		
Sore throat	18 (47)	14(35)		
Headache	20 (56)	8(21)**†	3.9	1.4-10.5
Myalgia	12 (28)	8 (20)		
Feverishness	24 (56)	14(35)*†	2.7	1.1-6.7
Patient characteristics:				
Vaccination	1 (2.5)	7 (18)*†	0.1	0.01-1
Vascular disease	0 (0)	3(7.5)‡		
Diabetes Mellitus	0 (0)	1 (2.5)		
COPD	1 (2.5)	4 (10)†	0.19	0.02-1.8

* P<=0.05 fisher's exact test ; **P<=0.01 fisher's exact test; † P<0.15

‡ P<0.15 not evaluable (all patients were pcr-negative)

Predictive value of criteria and clinical presentation. Seventy-nine out of 81 received questionnaires could be evaluated. Clinical features of 79 patients with clinical illness during this

period were compared with viral detection of influenza virus A by PCR (Table 2). Vaccination for influenza virus was significantly correlated with a negative outcome for influenza virus infection (P=< 0.05). Cough as a presenting symptom was significantly correlated with influenza A virus infection compared with the group of patients of which other respiratory viruses or no viral pathogen could be detected (P=< 0.01, positive predictive value (PPV) 57%, negative predictive value (NPV) 90%). Headache at onset of symptoms and feverishness at onset of symptoms were also positively correlated with influenza A virus infection (P<=0.05, PPV 71%, NPV 61% and PPV 63%, NPV 61% respectively). No other relations between clinical features and positive PCR could be found. Variables with a P<0.15 (period of increased influenza activity, cough, hoarseness, feverishness, headache at onset of symptoms, chronic obstructive pulmonary disease, vascular disease, and vaccination for influenza virus) were combined in a logistic regression model. Stepwise deletion of variables showed the best model with the combination of period of increased influenza activity, cough, headache at onset, feverishness at onset and vaccination status with a PPV of 75% and a NPV of 80%.

Table 3 Comparison of NIVEL criteria, ICHPPC-2 criteria, and GPs opinion

	PPV(%)	**NPV(%)**	**RR**	**95% CI**
NIVEL criteria	52	na	na	na
ICHPPC-2 criteria	54	85	2.4	0.3-18.3
GPs	76	75	6.8	1.4-33.3

na = not applicable, NIVEL criteria are equivalent to inclusion criteria

All of the patients met the NIVEL-criteria for ILI. Fifty-two percent (41/79) were infected with influenza virus. Seventy-two of the patients met the criteria of ICHPPC-2. The criteria of ICHPPC-2 showed a PPV of 54% and a NPV of 85% (Table 3). The GP's were asked to fill in their presumed aetiology of illness of the patients (influenza, other respiratory virus, and no viral pathogen). There was a significant correlation between the opinion of GP's and the outcome of pathogen in case of influenza virus infection (P<=0.01, PPV 76%, NPV 75%).

DISCUSSION

The results of our study on the complex of symptoms typical for influenza virus infection demonstrate a positive predictive value of 75% and a negative predictive value of 80% for the combination of cough, headache at onset, feverishness at onset, and vaccination status during the period with increased influenza activity. The GP's opinion on the viral aetiology of infection showed a PPV of 76% and a NPV of 75%. By using PCR an increase is seen in the detection of the viral agents responsible for the symptoms of disease.

Few studies have been done to evaluate the clinical presentation of respiratory virus infection. Govaerts et al. found in their study on the predictive value of influenza symptomatology in the elderly a predictive value of 44% of the complex of fever, acute onset and cough [10]. Our study is limited by the small group of patients in different age groups that only represents patients who visit their GP. It is therefor difficult to draw strong conclusions. The limited number of patients above 65 years can partly be explained by the high influenza vaccination coverage of almost 90% in this age group with a medical condition [23]. In this study the most outstanding symptom correlated with influenza virus infection was cough which confirms the results of other studies [10,15,16]. More of importance however, is the period in which the influenza epidemic is seen, which stresses the importance of surveillance networks. During the yearly period of increased influenza activity the practitioner's intuition of which case was indeed influenza was accurate. Based on experience, the GP's are more likely to interpret better the weight of symptoms of the presenting patients. Results are possibly biased due to two facts. First of all, the group of participating GP's was small and may not be representative. Secondly, the partaking physicians used a trial protocol, which made them conscious about making the correct diagnosis and also may have led to a more stringent application of diagnostic labels than usual, resulting in a high overall predictive value of the GP's opinion.

The small number of patients included by the GP's is explained by two facts. First of all, the 1997/1998 winter season was a very mild influenza season in the Netherlands compared to other years. According to the NIVEL surveillance there were 7 weeks of increased influenza activity (>5/10.000 inhabitants with ILI) and the epidemic threshold was not exceeded [14]. Another reason was the stringent inclusion criteria: to be able to perform sensitive confirmatory laboratory diagnosis we only included the patients that presented at the GPs within 48 hours of onset of symptoms. This group of patients presenting within 48 hours is also the target group for possible intervention with antiviral agents. Most patients in the Netherlands tend to consult their GP in a later stage of illness since the Netherlands General Practitioners (NHG) Standard advises to see patients when symptoms continue or worsen after 5 days of illness [8].

Laboratory diagnosis of influenza virus by PCR was more sensitive compared to culture or rapid culture. The fact that other studies have mainly used culture, serology or antigen testing might have resulted in underestimation of influenza [4,10,15,16]. We have therefore chosen this method as a gold standard instead of the less sensitive isolation of influenza virus by culture. Although numerous studies have been performed to compare different laboratory diagnostic methods, including PCR, most of these studies do not take into account the problems of transport of the specimen from general practice to the laboratory [5-7]. Ideally, transportation of the samples should take place at 4°C. Practically, samples are send by mail, overnight and at room temperature. The low recovery rate by culture in our study is very likely the result of viral inactivation during transport.

From our study we can conclude that using either the ICHPPC criteria or the sentinel criteria does not distinguish satisfactory between influenza and other viruses/pathogens causing these symptoms.

Intensification of the surveillance networks and notification of the results to GP's is one of the most powerful tools to diagnose influenza virus infection since during the influenza season it seems to be less difficult to distinguish influenza from other respiratory virus infections. It would be interesting to look at a larger scale, because besides intensive virological sampling by a surveillance network, clinical scoring could be a useful diagnostic tool at hand for clinicians, especially when treating for influenza virus infection.

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Chapter 4

Simultaneous detection of influenza A and B viruses using real-time quantitative PCR

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ABSTRACT

Since influenza viruses can cause severe illness, timely diagnosis is important for an adequate intervention. The available rapid detection methods either lack sensitivity or require complex laboratory manipulation. This study describes a rapid, sensitive detection method that can be easily applied in routine diagnostics. This method simultaneously detects influenza A and B viruses in the specimens of patients with respiratory infections using a Taqman based real-time PCR assay. Primers and probes were selected from highly conserved regions of the matrix protein of influenza virus A and the hemagglutinin gene segment of influenza virus B. The applicability of this multiplex PCR was evaluated on 27 influenza virus A and 9 influenza B virus reference strains and isolates. In addition, the specificity of the assay was assessed using 8 reference strains of other respiratory viruses (parainfluenza viruses 1-3, respiratory syncytial virus long strain, rhinoviruses 1A and 14, and coronaviruses OC43 and 229E) and 30 combined nose and throat swabs from asymptomatic subjects. Electron microscopy (EM)-counted stocks of influenza A and B viruses were used to develop a quantitative PCR format. Thirteen copies of viral RNA were detected for influenza A virus and 11 copies for influenza B virus equaling 0.02 $TCID_{50}$ and 0.006 $TCID_{50}$ respectively. The diagnostic efficacy of the multiplex Taqman PCR was determined by testing 98 clinical samples and showed that this real-time PCR technique was more sensitive than the combination of conventional viral culture and shell vial culture.

INTRODUCTION

Influenza virus infection is a highly contagious respiratory disease that can spread easily and is responsible for considerable morbidity and mortality each year. Elderly and compromised individuals are especially at risk of developing severe illness and complications. Rapid diagnosis, therefore, is important not only for timely therapeutical intervention, but also for the identification of a beginning influenza outbreak. Recently published results of clinical trials using new anti-influenza compounds, the neuraminidase inhibitors, demonstrated that these drugs are effective against influenza A and B viruses and are most effective when administered early upon emerging symptoms [1,7,9]. With the development of such new treatment options, rapid detection methods become even more desirable.

Virus isolation via cell culture, shell vial culture, antigen detection and serology are the methods currently used for the laboratory diagnosis of influenza viruses. Each of these methods, however, has its limitations. For example, although virus isolation via cell culture can be a robust and sensitive method for the detection of limited numbers of viable virions, it is

labor-intensive and depends on optimal sample transport for sensitive virus isolation. Moreover, since the concentrations of viable virus can decline rapidly after the first days of the infection, the virus can become undetectable by culture in the later course of infection [8]. Finally, the results from cell culture are generally obtained too late for adequate intervention.

Alternative diagnostic techniques, such as viral antigen detection (immunofluorescence and enzyme immunoassay techniques) and shell vial culture on the other hand, show results much more quickly, but are generally less sensitive than conventional cell culture [5,12,15,18,20].

To overcome this lack of sensitivity and also obtain rapid diagnostic results, PCR techniques were developed for the specific detection and subtyping of influenza viruses. They have proven to be very sensitive and specific, but unfortunately are often difficult to implement in a routine diagnostic setting and still require time-consuming sample handling and post-PCR analysis [2,4,6]. Needless to say, better techniques are still needed.

Here, we describe a multiplex Taqman-based real-time PCR assay for the rapid and simultaneous detection of influenza viruses (influenza virus A, influenza virus B, or both) in clinical specimens. We also compare this real-time PCR assay to conventional culture methods and to an in-house nested PCR assay. The method can generate results within 4-5 hours and does not require any post PCR handling [10,11,14]. Moreover, the assay can be used for direct virus quantification and can be easily implemented in routine viral diagnostic testing.

MATERIALS AND METHODS

Virus stocks. Influenza A/Port Chalmers/1/73 (H3N2), influenza B/Lee/40 and parainfluenza viruses 1-3 were obtained from the American Type Culture Collection (Rockville, MD, USA). Influenza A and B virus reference strains and isolates, and reference strains of rhinovirus 1A, rhinovirus 14, respiratory syncytial virus long strain, coronavirus OC43, and coronavirus 229E were kindly provided by the Laboratory for Virology, National Institute of Public Health and the Environment (RIVM) (Bilthoven, the Netherlands).

Virus particles count. Purified human influenza virus A/PR/8 (H1N1) (virus particles were counted by electron microscopy (EM)), was ordered from Advanced Biotechnologies Incorporated (ABI) (Columbia, MD, USA). Influenza virus A/Texas/36/91 (H1N1), influenza virus A/Port Chalmers/1/73 (H3N2), and influenza B/Lee/40 were propagated at 33ºC on tertiary rhesus monkey kidney (tRMK) cells pre-treated with Eagle minimal essential medium (Bio Whittaker) supplemented with streptomycin, penicillin, amphotericin B, and 0.01% trypsin. After the development of a cytopathic effect, cells and supernatant were harvested and frozen at –70ºC. The viral particle count of each stock was then determined by quantitative EM (ABI).

Clinical specimens. Combined nose and throat swabs or nasal washes were taken from individuals presenting with upper- or lower respiratory symptoms. Some of these specimens were taken at regional general practices (GPs) participating in a study to evaluate the efficacy of influenza vaccination. The other clinical samples were obtained from patients presenting with respiratory illnesses at the University Medical Center Utrecht in the period 1998-1999. Routine diagnostic logistics were used for the sample transportation from the GPs to the laboratory as well as for the sample transportation from the outpatient clinic to the laboratory. The samples that were sent by mail were left at room temperature for a maximum of 24 h. The samples from the outpatient clinic were sent to the laboratory within 2 h. All of the samples were transported in 5 ml of virus transport medium. Nasal wash specimens and swabs were vortexed for 10 s and centrifuged at 2,000 x g for 15 min. One ml of the supernatant was used directly for virus culture. The remaining material was stored at –70ºC until RNA extraction.

Virus isolation and growth. Confluent tRMK cells were inoculated with 100 µl of each clinical sample. After absorption for 1 h at room temperature, the inoculum was removed and 5 ml fresh medium containing Eagle minimal essential medium (Bio Whittaker) supplemented with 0.02 M Hepes, 0.075% bicarbonate, 100 E/ml penicillin/streptomycin, 25 E/ml nystatin (Gibco), 0.2 M glutamine (SVM), and 0.01% trypsin (SVM) was added. The cultures were then incubated at 33ºC on roller drums and examined twice weekly for 10 days for cythopatic effect. Regular testing for hemadsorption was performed using a 0.25% guinea pig erythrocyte suspension. Positive cultures were identified by immunofluorescence with commercial monoclonal antibodies (Dako Imagen) for influenza A and B viruses and parainfluenza viruses 1-3. Further subtyping of the strains was performed at the National Reference Center for Influenza in Rotterdam, the Netherlands.

After 2 days of culture, usually before a cytopathic effect was noticed, rapid antigen testing was performed by immunofluorescence with commercial monoclonal antibodies (Dako Imagen) for influenza A and B viruses (shell vial culture). The supernatants of the clinical specimens were also cultured on other tissue cell lines (R-Hela cells and HEP-2c cells) for the detection of other respiratory viruses.

Viral genomic RNA isolation and cDNA synthesis. RNA extraction was performed according to the method described by Boom et al. [3]. Briefly, 10-100 µl respiratory specimen, tissue culture supernatant or EM-counted virus stock were mixed with 900 µl lysis buffer and 50 µl silica and incubated for 10 min at room temperature in order to bind the nucleic acid to the silica particles. Unbound material was then removed by several washing steps. The RNA was then eluted either in 100 µl 40 ng/µl polyA RNA before performing a one-tube reverse transcription (RT)-PCR [13] or in 100 µl RNase-free water before cDNA synthesis.

cDNA was synthesized by using MultiScribe^{TM} Reverse Transcriptase and random hexamers (both PE Applied Biosystems). Each 50 µl reaction contained 10 µl eluted RNA, 5 µl 10x RT buffer, 5.5 mM $MgCl_2$, 500 µM (each) dNTPs, 2.5 µM random hexamer and 0.4 U/µl RNase inhibitor (all PE Applied Biosystems). After incubation of 10 min at 25ºC, reverse transcription was carried out for 30 min at 48ºC followed by RT inactivation for 5 min at 95ºC. The cDNA was then stored at –70ºC before further use.

Qualitative PCR. A multiplex nested PCR was performed for influenza A and B viruses. A one-tube RT-PCR was followed by a second (nested) amplification. First-round amplification primers and nested

primers were selected in conserved regions of the matrix protein for influenza virus A (first-round primer set FLU-1 5' CAGAGACTTGAAGATGTCTTTGC3', FLU-2 5' GGCAAGTGCACCA-GCAGAATAACT 3' and the second-round primer set FLU-3 5' GACCRATCCTGTCACCTCTGACT 3', FLU-4 5' ATTTCTTTGGCCCCATGGAATGT 3') and the hemagglutinin gene segment of influenza virus B (FLUB-5 5' GAATCTGCACTGGGATAACATC 3', FLUB-8 5' TTTGTTCTGTC-RATGCATTATAGG 3' and the inner primer set FLUB-2 5' TCTCATTTTGCAAATCTCAAAGG 3', FLUB-3 5' TCRTGGAGTATTGAARCTTTTGC 3'). The RT-PCR and nested PCR conditions we applied were as described by M. Nijhuis et al.[13] using a PE 9600 Thermocycler (Perkin Elmer). PCR products were visualized on an ethidium bromide-stained agarose gel using UV illumination. A 5 µl 100 base-pair marker was used, to control fragment lengths.

Table 1. Selected primers and probes for the Taqman amplification of viral RNA from influenza A and B viruses

Influenza virus	**Primer/probe**	**Sequence**	**position**[a]
type (target)			
A (M gene)	INFA-1	5' GGACTGCAGCGTAGACGCTT	217-236
	INFA-2	5' CATCCTGTTGTATATGAGGCCCAT	382-405
	INFA-3	5' CATTCTGTTGTATATGAGGCCCAT	277-300
	INFA-probe	5' CTCAGTTATTCTGCTGGTGCACTTGCCA	349-376
B (HA gene)	INFB-1	5' AAATACGGTGGATTAAATAAAAGCAA	970-995
	INFB-2	5' CCAGCAATAGCTCCGAAGAAA	1119-1139
	INFB-probe	5' CACCCATATTGGGCAATTTCCTATGGC	1024-1050

[a] Primer and probe positions for influenza A viruses correspond to the M gene of A/Port Chalmers/1/73 (H3N2) and A/Texas/36/91 (H1N1) and for influenza B viruses to the HA gene of B/Lee/40

Real-time quantitative PCR. Primers and probes for both influenza A and B viruses were selected using primer express software (PE Applied Biosystems) and based on the genomic regions of high conservation of various subtypes and genotypes of influenza A virus (matrix protein gene) and influenza B virus (hemagglutinin gene segment). The exact primers and probes were chosen after the sequence comparison of 39 influenza A strains and 44 influenza B strains. Probes were obtained without runs of identical nucleotides to avoid non specific interactions, with no G s on the 5' end, and with a melting temperature of 69ºC (10ºC above the melting temperature of the primers to ensure full hybridization of the probe during primer extension). Moreover, primers and probes were tested for possible interactions to make sure they could be used together in a multiplex assay. Forward and reverse primers (INFA-1, INFA-2, INFA-3, INFB-1, and INFB-2) and probes (INFAp1/3 and INFBp1/2) are shown in Table 1. Two forward primers were selected for influenza A virus with a different nucleotide from the 4th base at the 5' end to ensure that all strains of influenza A virus could be detected. Both fluorogenic probes for influenza A and B viruses consisted of oligonucleotides with a 5'

reporter dye FAM (6-carboxy-fluorescein) and a 3' quencher dye TAMRA (6-carboxy-tetramethyl-rhodamine). A 25 µl PCR reaction was performed using 5 µl cDNA, 12.5 µl Taqman universal PCR master mix containing ROX as a passive reference (PE Applied Biosystems), 900 nM of each influenza A primer, 300 nM of each influenza B primer, and 100 nM of each probe. Amplification and detection were performed with the ABI Prism 7700 sequence detection system under the following conditions: 2 min at 50ºC to require optimal AmpErase UNG activity, 10 min at 95ºC to activate AmpliTaq Gold DNA polymerase followed by 45 cycles of 15 s at 95ºC and 1 min at 60ºC.

During amplification, the ABI Prism sequence detector monitored real-time PCR amplification by quantitatively analyzing the fluorescent emission. The reporter dye FAM signal was used against the internal reference dye ROX to normalize for non PCR-related fluorescence fluctuations occurring well-to-well. The Ct (threshold cycle) represented the refractional cycle number at which a positive amplification reaction was measured and was set at 10 times the standard deviation of the mean baseline emission calculated for PCR cycles 3 to 15.

RESULTS

Sensitivity. The sensitivity of the multiplex assay was determined in two ways: 1) by a virus infectivity assay and 2) by counting the viral particles using EM. Influenza A/PR/8/34 (sucrose gradient-purified) and influenza B/Lee/40 were first EM-counted and subsequently titrated by serial dilution. The 50% tissue culture-infective dose ($TCID_{50}$) values for the two strains, calculated by the Kärber-formula, were 1.8 $x10^9$/ml and 2.0 $x10^9$/ml, respectively, corresponding to 9 $x10^{11}$ viral particles (vp) and 3.3 x 10^{12} vp.

The tenfold serial diluted concentrations of the two strains were then amplified using the multiplex Taqman assay. Eleven vp of influenza B/Lee/40 and 13 vp of influenza A/PR/8/34 could be detected in both the multiplex Taqman assay as well as in the separate Taqman assays for influenza A and B viruses (Fig. 1). This level of sensitivity correlated with 0.02 $TCID_{50}$ of influenza A/PR/8/34 and 0.006 $TCID_{50}$ of influenza B/Lee/40.

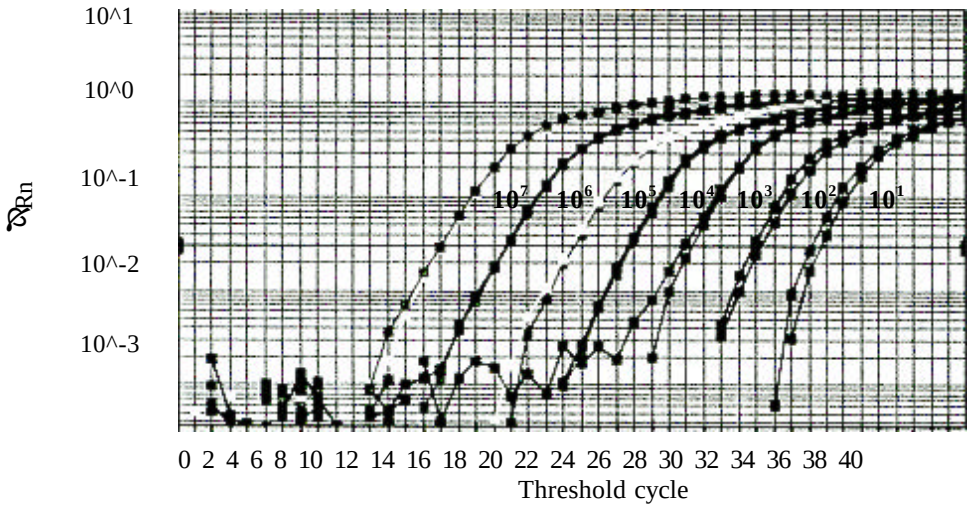

Figure 1. Standardization of influenza B in the multiplex Taqman assay. Serial dilutions were made using the EM-counted influenza B/Lee/40 virus stock. A minimum of ±10 copies RNA could be detected after 40 cycles. The intensity of fluorescence is given on the y-axis.

Specificity. The specificity of the multiplex Taqman PCR was assessed by testing reference strains of subtypes of influenza A virus H1N1 (A/Singapore/6/86, A/Taiwan/1/86, A/Texas/36/91, A/Bayern/7/95, A/PR/8/34, and NIB-39rec Bayern), H2N2 (A/Singapore/1/57, A/Japan/307/57 and A/England/1/66) H3N2 (A/Hongkong/1/68, A/Philadelphia/2/82, A/Shangdong/9/93, A/RESVIR, A/Sydney/5/97, and A/Port Chalmers/1/73), influenza B virus (B/Yamagata/16/88, B/Lee/40, B/Panama/45/90, and B/Singapore/222/79), and a variety of other respiratory viruses (rhinovirus 1A, rhinovirus 14, respiratory syncytial virus (long strain), coronaviruses OC 43 and 229E, and parainfluenza viruses 1-3). Five H1N1, 7 H3N2, and 5 influenza B patient isolates were also tested. All of the influenza virus strains but none of the other respiratory viruses were detected. In addition nose and throat swabs taken from 30 asymptomatic subjects during the winter season were analyzed by the multiplex Taqman PCR to assess the possibility of false-positive results; none of the samples gave a positive signal.

Table 2. Comparison of culture/shell vial culture, multiplex Taqman PCR and nested multiplex PCR for the detection of influenza A and B viruses in clinical specimens (*n*=98)

	No. of positive samples (%)	No. of negative samples (%)
Culture/shell vial culture	22(12%)	76(88%)
Multiplex Taqman PCR	40(41%)	58(59%)
Influenza A Taqman PCR	36(37%)	62(63%)
Influenza B Taqman PCR	4(4%)	94(96%)
Nested multiplex PCR	44(45%)	54(55%)

Comparison of Taqman PCR, shell vial culture and conventional culture to nested RT-PCR in clinical specimens. A total of 98 clinical specimens were collected during the 1998-1999 and 1999-2000 winter seasons. Eighty of the samples were sent by mail at room temperature, whereas 18 of the samples were transported to the lab immediately at 4ºC. The samples were analyzed for influenza A and B viruses using multiplex nested PCR, multiplex Taqman PCR, cell culture, and shell vial culture (Table 2). All of the nested RT-PCR positive samples were subsequently used in a sensitivity analysis. When the results of the multiplex Taqman PCR and the combined results of conventional cell culture and shell vial

culture were compared with the nested PCR, overall sensitivities of 88% and 51% respectively were found. The 18 samples that were transported at 4ºC showed sensitivities of 83% for multiplex Taqman PCR and 44% for the conventional culture and/or shell vial culture respectively. The 80 samples that were sent by mail at room temperature showed sensitivities of and 96% for multiplex Taqman PCR and 57% for conventional culture and/or shell vial culture.

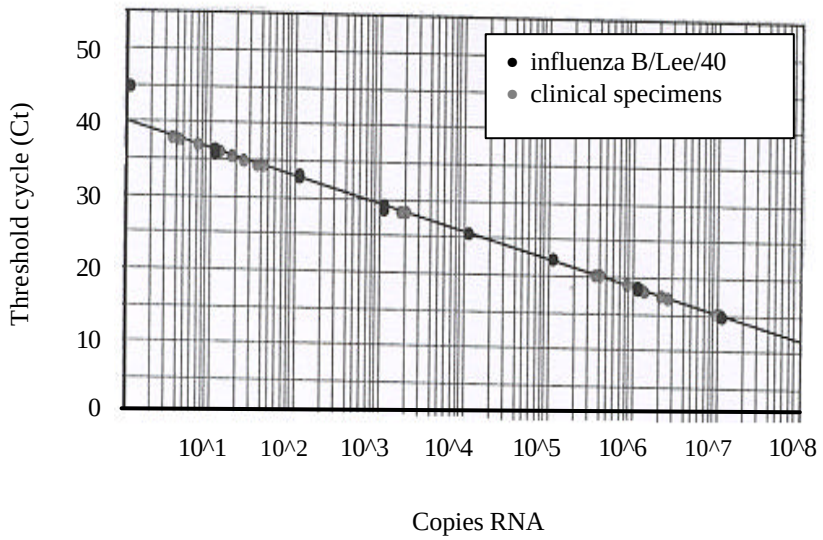

Figure 2. Standard curve generated by the analysis of known amounts of viral RNA of influenza virus B/Lee/40 with the multiplex Taqman PCR. Unknown quantities of virus in clinical specimens are related to the standard curve.

Longitudinal follow-up. Six patients infected with influenza virus (2 with influenza B virus and 4 with influenza A virus (H3N2)) were followed during their infections. A total of 30 nasal washes were obtained on days 1-3, 7, and 14 after the presentation of influenza-like symptoms. The number of viral RNA copies in the clinical samples was determined by extrapolation to a standard curve generated upon amplification of serial dilutions of the EM-counted virus stocks (A/PR/8/34 and B/Lee/40) (Fig. 2). Using the multiplex Taqman PCR, we were able to detect and quantify influenza virus in nasal washes up to 7 days after the initial presentation of influenza-like symptoms in 4 patients, as shown in Fig. 3. Using conventional culture, we could only detect virus on day 7 in one patient. The multiplex Taqman PCR was also much more sensitive in the detection of influenza A and B viruses than culture

and/or shell vial culture: 20/30 (66%) specimens were positive using the multiplex Taqman PCR, while 11/30 (35%) specimens were positive using tissue cell culture and/or shell vial culture.

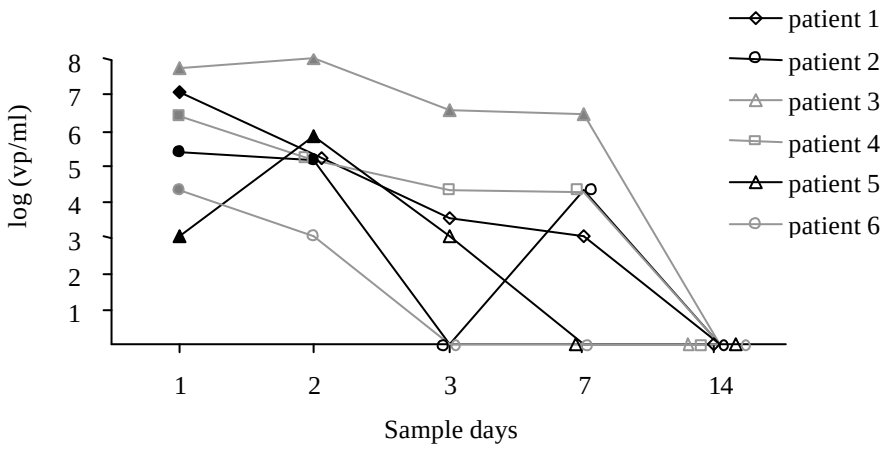

Figure 3. Longitudinal follow-up of 6 patients with either influenza A virus (patient 3-6) or B virus infection (patient 1 and 2). Quantitative analysis was performed using the multiplex Taqman PCR. The clinical samples were related to the standard curve. The filled symbols represent the clinical specimens that were also positive by culture and/or shell vial culture.

DISCUSSION

Our findings demonstrate that the multiplex Taqman PCR is a sensitive and specific method for the simultaneous rapid detection of influenza A and B viruses. In fact, we were able to detect as little as 0.02 $TCID_{50}$ for influenza virus A and 0.006 $TCID_{50}$ for influenza virus B, corresponding to approximately 10 viral RNA copies.

For epidemiological reasons, it is may be important to type and subtype the influenza strains. In recent studies typing and subtyping of influenza virus strains has been performed using (multiplex) RT-PCR [6,16,19]. This type of analysis, however, is time-consuming either because (sub)type-specific PCRs need to be performed or because the post-PCR analysis is complicated.

The multiplex Taqman PCR described here, allows the extremely rapid and accurate diagnosis of both types of influenza viruses within 4-5 hours. Our type specific probes, for example, can

be labeled with different fluorogenic dyes to distinguish between influenza A and B viruses because the ABI PRISM 7700 sequence detection system has the capability of detecting multiple dyes with distinct emission wavelengths [17]. Then, sequential Taqman PCRs using subtype-specific primers can be performed to subtype influenza A viruses in detail [16].

Besides being rapid, this method also has the advantage of a standardized protocol that can easily be applied for other respiratory viruses: the Taqman PCR can be performed under uniform amplification conditions, thereby using target specific primer and probe sets. In addition, the procedure is less complicated than other RT-PCR methods and the chances of contamination are minimized because there is no post-PCR handling of the samples.

The multiplex Taqman PCR was more sensitive than the standard conventional culture/shell vial culture, i.e. the multiplex Taqman PCR detected influenza viruses at lower concentrations. The low recovery rate with culture techniques is usually explained by viral inactivation caused by the transportation of the samples. However, in this study the transport conditions did not affect the sensitivity of conventional culture, although the number of tested clinical specimens was small.

In order to correct for false-positive results, we took samples not only from symptomatic patients, but also from asymptomatic individuals during the same influenza season. Since none of these latter samples contained influenza viral RNA, the positive results from the multiplex Taqman PCR, which were confirmed by nested PCR, can be considered true-positives.

The follow-up of the six symptomatic patients showed that influenza can be detected up to 7 days after infection using the multiplex Taqman PCR, a period when most of the patients were still clinically ill. In contrast, influenza virus could only be isolated using conventional virus culture, from the first one or two days in the majority of these patients.

We were able to quantify our PCR technique using serial dilutions of EM-counted stocks of influenza A and B viruses a standard curve could be generated in the multiplex Taqman PCR, and as such created a quantitative format of the assay. Even though influenza virus infection usually only persists for one week, quantification might be a useful tool in evaluating the effects of antiviral therapy.

In conclusion, we have developed a rapid, highly sensitive and specific quantitative real-time PCR for the simultaneous detection of influenza A and B viruses. Results can be obtained within a few hours, thus allowing time for adequate clinical management and the evaluation of antiviral therapy.

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Chapter 5

Applicability of a novel real-time quantitative PCR assay for the diagnosis of respiratory syncytial virus infection in immunocompromised adults

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ABSTRACT

Respiratory syncytial virus (RSV) accounts for the majority of respiratory virus infections with high mortality rates in immunocompromised patients with hematologic malignancies. The available methods for the rapid detection of RSV by antigen detection or PCR either lack sensitivity, require complex laboratory manipulation, or have not been evaluated in this patient population. To asses the applicability of a Taqman-based real-time PCR technique for the detection of RSV A and B in immunocompromised adults, we developed a rapid, sensitive detection method that simultaneously detects RSV A and B viruses and can be applied in routine diagnostics. The specificity of the assay was assessed using a panel of reference strains of other respiratory viruses and RSV. Electron microscopy (EM)-counted stocks of RSV A and B were used to develop a quantitative PCR format. Eleven copies of viral RNA could be detected for RSV strain A Long and 14 copies for RSV strain B 9320 corresponding to 0.86 $TCID_{50}$ and 0.34 $TCID_{50}$, respectively. The assay was evaluated on 411 combined nose- and throat swabs derived from immunocompromised adults with- and without signs of respiratory tract infection. The diagnostic efficacy of the Taqman PCR determined on the clinical samples showed that this real-time PCR technique was substantially more sensitive than the combination of conventional viral culture and shell vial culture. None of the clinical specimens derived from patients without signs of respiratory illness were found positive for RSV by real-time Taqman PCR.

INTRODUCTION

Respiratory syncytial virus (RSV) has since long been recognized as a major cause of respiratory tract infection in infants and young children [14]. More recent studies have shown that those at risk for developing serious disease following RSV infection also include the elderly, adults with underlying cardiopulmonary disease, and the severely immunocompromised [3,4,7,17,21,25,26]. Compared with pneumonias caused by other respiratory viruses, RSV pneumonias are associated with the highest mortality in bone marrow transplant recipients and leukemia patients [25,27].

Children are known to shed RSV in high titers for up to several weeks, whereas shedding of virus in adults and the elderly is presumed to be of relatively low titer and short duration [8]. Consequently, laboratory techniques, such as conventional cell culture and antigen detection assays that are suitable for diagnosis in young children, are hampered by lack of sensitivity in the elder patient [6]. Even serologic analysis may not always be reliable in certain patient groups because of their impaired immune response.

To overcome this lack of sensitivity and to obtain more rapid diagnostic results, a number of different PCR techniques has been developed [16,22]. Earlier, we described a substantial increase in the detection of respiratory viruses involved in pneumonia in patients with hematologic malignancies with the use of nested PCR methods [20]. Reverse transcription-PCR (RT-PCR) has also been proven to be

more sensitive than viral culture in adults with or without cardiopulmonary disease with respiratory illness, and are a well-considered alternative for the rapid diagnosis of RSV infection [5].

However, RT-PCR and nested-PCR are difficult to implement in a routine diagnostic setting because they need time-consuming sample handling and post-PCR analysis, often requiring specific detection methods, and posing serious hazards for amplification product carryover. The current development of real-time-PCR methods seems to overcome these problems [10,19,24]. A recent study used real-time RT-PCR and immunofluorescence (IF) in nasopharyngeal aspirates derived from children containing moderate to low levels of RSV and showed more or less equal sensitivities of both methods indicating that the advantage of real-time PCR in children consists primarily of the automated analysis of results and the possibility of direct virus quantification [10].

RSV infection can have devastating consequences in patients treated for leukemia [26]. Since specimens derived from children are known to contain high viral loads, and the recovery rates for RSV are generally lower in adult patients, we were interested to see whether the increased sensitivity of real-time PCR would also provide more clinical benefit in immunocompromised adults.

The purpose of our study was to asses the applicability of a Taqman-based real-time PCR technique for the detection of RSV A and B in immunocompromised adults. Therefore, we compared real-time PCR with conventional cell culture, shell vial culture and our in house nested PCR. To determine the clinical value of the assay, specimens were taken from symptomatic as well as asymptomatic patients.

MATERIALS AND METHODS

Virus stocks. RSV A Long was propagated on human embryonic lung fibroblast cells and RSV B 9320 was propagated on HEp-2 cells at 35°C in Eagle's minimal essential medium supplemented with 0.01 M HEPES, 0.084%bicarbonate, 100 U/ml of penicillin and streptomycin, 0.625 µg/ml fungizone and 0.2 M glutamine (SVM, Foundation for the Advancement of Public Health and Environment, Bilthoven, The Netherlands). After development of a cytophatic effect, the supernatant was harvested and the virus particle count of each stock was determined by quantitative electron microscopy (EM) (Advanced Biotechnologics Incorporated, Columbia, Md). A previously described, well defined panel of various respiratory viruses provided by the Laboratory for Virology, National Institute for Public Health and the Environment (RIVM, Bilthoven, The Netherlands) was used to determine the specificity of the real-time quantitative PCR [19]. In addition, 16 strains of wild-type RSV obtained from successive seasons were tested.

Clinical specimens. From October 1999 through November 2002 a prospective surveillance study was carried out within a group of patients (n=73) who underwent autologous or allogeneic stem cell transplantation. Combined nose and throat (NT) swabs were collected at set time-points and during episodes of upper or lower respiratory tract symptoms. The majority of the samples were obtained from patients who participated in this study. The other clinical samples were collected from patients (n=17) known with hematologic malignancies who had signs of respiratory tract infection. In addition, 30 combined NT swabs from healthy, asymptomatic volunteers were collected. All NT samples were placed on ice immediately after collection and transported in 5 ml of virus transport medium to the laboratory within 2 h. There, the samples were vortexed for 10 s and

centrifuged at 2,000x *g* for 15 min. One ml of the supernatant was used directly for virus culturing. The remaining material was stored at -70°C until RNA extraction.

Diagnostic methods for the routine detection of RSV. For shell vial cultures, 100 µl of clinical specimen was inoculated on tertiary rhesus monkey kidney cells grown in flat bottom tubes, centrifuged for 1 hour at 2,000x *g* and incubated for 2 days at 33°C. Then, usually before a cytopathic effect could be noticed, cells were fixed and stained with virus-specific monoclonal antibodies (Dako Imagen). Immunofluorescence microscopy was used to detect RSV. For routine viral cultures, 100 µl of each clinical specimen was inoculated on HEp-2 cells, R-HELA cells and tertiary rhesus monkey kidney cells and incubated at 33°C for a maximum of 10 days with 100 of each clinical sample. In cultures showing a cytopathic effect, virus was identified by immunofluorescence with commercial monoclonal antibodies (Dako Imagen) for RSV.

Viral RNA isolation and cDNA synthesis. RNA extraction was performed using the MagnaPure LC Total Nucleic Acid Kit (Roche Diagnostics, Mannheim, Germany). Briefly, 10-100 µl of clinical specimen or EM-counted virus stock was mixed with lysis buffer and proteinase K and subsequently incubated with magnetic particles to allow binding of the nucleic acids. Unbound material was removed by several washing steps. The RNA was then eluted either in 100 µl 40 ng/µl polyA RNA before performing a one-tube reverse transcription (RT)-PCR [13] or eluted in 100 µl elution buffer and directly used for cDNA synthesis and real-time Taqman PCR.

The isolated viral RNA was reverse transcribed using MultiScribe^{TM} Reverse Transcriptase and random hexamers (Taqman Reverse transcription Reagents, ABI). Each 50 µl reaction contained 10 µl eluted RNA, 5 µl 10x RT buffer, 5.5 mM $MgCl_2$, 500 µM of each of the deoxynucleoside triphosphates, 2.5 µM random hexamer and 20 U of RNase inhibitor (all from Applied Biosystems International). The cDNA synthesis was performed as described previously [19] and the cDNA was stored at -70°C until real-time Taqman PCR.

In-house nested PCR. A nested PCR was performed for RSV A and B viruses. A one-tube RT-PCR was followed by a second (nested) amplification. First-round amplification primers and nested primers were defined in the N gene (first-round primer set RS-1 5'-GGATTGTTTATGAATGCCTATGGT-3', RS-2 5'-TTCTTCTGCTGTYAAGTCTARTACAC-3' and the second-round primer set RS-3 5'-GGATTCTACCATATATTGAACAA-3', RS-4 5'-CTRTACTCTCCCATTATGCCTAG-3').

Table 1. Selected primers and probes for the Taqman amplification of viral RNA from RSV A and B viruses

RSV type (target)	RSV type (target)	Sequence	Nucleotide position[a]
A (N gene)	RSA-1	5' AGATCAACTTCTGTCATCCAGCAA	1137
	RSA-2	5' ATTGATACTCCTAATTATGATGTGC	1192
	RSA-probe	5' CACCATCCAACGGAGCACAGGAGAT	1164
B (N gene)	RSB-1	5' AAGATGCAAATCATAAATTCACAGGA	1248
	RSB-2	5' CACTATAAAGATACTTAAAGATGCTGGATATCA	1318
	RSB-probe	5'AGGTATGTTATATGCTATGTCCAGGTTAGGAAGGGAA	1279

[a] Primer and probe positions are given according to the RSV A sequence (Genbank accession number M11486) and the RSV B sequence (Genbank accession number D00736).

The RT-PCR and nested PCR conditions were applied as described by M. Nijhuis et al. [13] using a PE 9600 Thermocycler (Perkin Elmer). PCR products were visualized on an ethidium bromide-stained agarose gel using UV illumination. A 100 base-pair marker was used to control fragment lengths.

Real-time Taqman PCR. Primers and probes for both RSV A and B viruses were selected using primer express software (PE Applied Biosystems) and were based on the genomic regions of high conservation of the N gene. To provide subgroup analysis, type specific primers and probes were chosen for RSV A and B. The forward and reverse primers (RSA-1, RSA-2, RSB-1 and RSB-2) and probes (RSA-probe and RSB-probe) used are shown in Table 1. Primers and probes were tested for possible interactions to make sure they could be used together in a multiplex assay. After optimization of primer and probe concentrations, samples were assayed in duplicate in a 25 ul reaction mixture containing 5 ul of cDNA, 12.5 ul 2xTaqMan Universal PCR Master Mix (PE Applied Biosystems), 900 nM of each forward primer, 900 nM of the reverse primers and 200 nM of each of the probes. The fluorogenic probes that can be labeled with different fluorogenic dyes were both labeled with the 5' reporter dye 6-carboxy-fluorescein (FAM) and the 3' quencher dye 6-carboxy-tetramethyl-rhodamine (TAMRA). Amplification and detection were performed as described previously [19].

During amplification, the ABI Prism sequence detector monitored real-time PCR amplification by quantitatively analyzing the fluorescence emissions. The reporter dye (FAM) signal was measured relative to the internal reference dye (ROX) to normalize for non PCR-related fluorescence fluctuations occurring from well to well. The threshold cycle number represented the refractional cycle number at which a positive amplification reaction was measured.

RESULTS

Specificity and sensitivity. The specificity of the real-time TaqMan PCR was assessed by testing a variety of other respiratory viruses (rhinovirus 1A and 14, coronaviruses OC43 and 229E, parainfluenza viruses 1 to 4, influenza virus B/Lee/40, influenza virus A/PR/8/34 and enterovirus CVA9 and CVA11) and a panel of 16 wild-type RSV strains circulating at consecutive seasons. All of the RSV strains but none of the other respiratory viruses were found positive by real-time Taqman PCR. No fluorescent signal was observed in 30 specimens collected from healthy adults without symptoms of respiratory infection.

The sensitivity of the assay was determined by comparing real-time PCR results with results from (i) EM particle counting and (ii) a virus infectivity assay. RSV A Long and RSV B 9320 were first counted by EM and subsequently titrated by serial dilution. The 50% tissue culture infective doses ($TCID_{50}$), calculated by the Kärber formula, were 1.0×10^6 and 3.2×10^5/ml, respectively, corresponding to 1.28×10^7 and 1.30×10^7 viral particles, respectively.

A 10-fold dilution series of the two strains was then amplified using the real-time PCR assay, indicating a detection limit of 11 particles of RSV A Long and of 14 particles of RSV B 9320 could be detected. This level of sensitivity correlated with 0.86 $TCID_{50}$ of RSV A Long and 0.34 $TCID_{50}$ of RSV B 9320.

Table 2. Comparison of conventional culturing, shell vial culturing, In-house nested PCR and real-time Taqman PCR for the detection of RSV in 411 clinical specimens

Method	No. of RSV positive samples	
	During period of symptoms (n=168)	During symptom-free period (n=243)
Conventional culture	4	0
Shell vial culture	0	0
In-house nested PCR	13	0
Real-time Taqman PCR	13	0

Detection of viruses in clinical samples. Taqman-based real-time RT-PCR has been shown to be a rapid, sensitive, and specific assay for the detection of RSV A and B viruses in children. To assess the applicability of a real-time PCR assay in immunocompromised adults, the assay was evaluated on combined nose and throat swabs collected from patients with hematologic malignancies. A total of 411 NT samples from 90 patients were tested by real-time Taqman PCR, an in-house nested PCR, conventional culture and shell vial culture. Of the 411 samples, 168 (41%) were obtained during an episode of suspected upper or lower respiratory tract infection and 243 (59%) were taken during an asymptomatic period. Overall, RSV was identified in a total of 13 (3.2%) specimens (Table 2). Of these RSV positive specimens, only 4 (31%) were detected by cell culture and none by shell vial culture, whereas all 13 (100%) were also detected by both the in-house nested PCR. All 4 culture positive samples were found positive by the Taqman based real-time PCR assay. Interestingly, none of the 243 samples taken during a symptom-free period were found to be positive for RSV by any of the applied methods. The Taqman based real-time PCR was found to be about 70% more sensitive than culture in this specific patient population. The number of viral RNA copies in the clinical samples was determined by extrapolation to a standard curve generated upon amplification of serial dilutions of the EM-counted virus stocks. Results of the quantification of the clinical specimens are shown in Fig. 1. Samples with a viral load above 10^8 viral particles/ml (threshold cycle below 30) could also be detected by virus culture. Surprisingly, one sample with a viral load of $1.4x10^5$ viral particles/ml could be detected by culture as well.

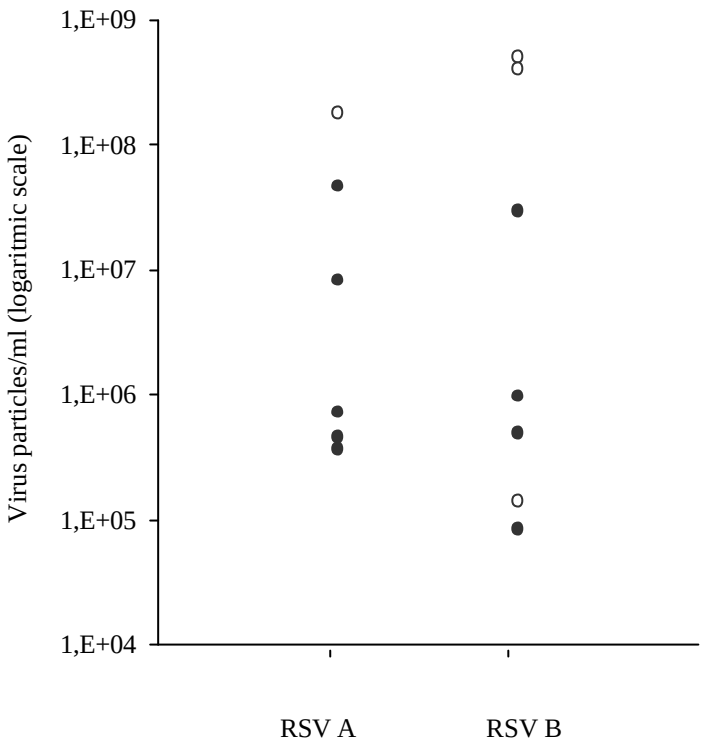

Figure 1. Amount of virus particles (vp) of RSV A or B that could be detected in the clinical specimens (n=13). The filled symbols (•) represent the clinical samples detected by real-time Taqman PCR only, whereas the unfilled symbols(o) represent the clinical samples detected by both virus culture and real-time Taqman PCR.

DISCUSSION

Our study shows that the Taqman real-time PCR can be used as a rapid and sensitive diagnostic tool for the detection of RSV in immunocompromised adults. The study confirms the lack of sensitivity of viral culture for RSV in the adult population. Moreover, our data indicate that the detection of RSV by nested PCR and Taqman real-time PCR is superior over shell vial culture as well.

The group which we studied, consisting of mainly immunocompromised patients who recently underwent a stem cell transplantation, is relatively small. We consequently acquired only a small proportion of RSV positive samples. The proportional contribution of RSV infection in our study does not differ however from other studies. Published reports on estimated frequencies of different respiratory viruses causing respiratory infection in the immunocompromised host showed that the contribution of RSV varies between 1.5% and 15 percent in two large epidemiological studies [12,27].

Both shell vial culture and conventional virus culture are well-established standard techniques that are used in routine laboratories for the detection of respiratory viruses in adults [11,15]. Rapid laboratory methods such as direct antigen detection are often used for point of care diagnosis of RSV infection in infants and children [18,23]. In adults, these methods have been shown to be unreliable, partly because of the sampling methods and partly because of the common believe that they tend to shed less virus

[2]. Our study shows a poor result for the conventional virus culture and the shell vial culture, although every effort was done to optimize sample handling such as sample transportation on ice and sample processing within 2 hours. It has been reported before that the use of RT-PCR in adults with respiratory illness can double the number of RSV infections detected compared to viral culture [5]. The type of specimen collection may be another explanation for the poor results on virus culture. In accordance with general experience we found that the majority of sometimes very ill, immunocompromised patients did not agree with the collection of nasal wash specimens. Therefore we decided to only evaluate combined NT-swabs that are generally found inferior to nasal wash specimens for the detection of RSV by viral culture. Our results indicate that conventional culture as well as shell vial culture might not be suitable for the identification of low viral loads. The majority of culture positive samples contained a viral load above 10^8 viral particles/ml (threshold cycle below 30) whereas viral loads in the lower ranges were mainly detected by the Taqman real-time PCR assay.

RT-PCR has proven to be a sensitive method for the detection of RSV infection in adults with respiratory illness [5]. Because PCR-based diagnostics provide an excellent potential for rapid diagnosis, with substantial consequences such as more rapid clinical intervention through therapy and infection control measures, their use has gained interest over the last couple of years. Yet, immunocompromised patients and children are known to shed virus for a long period of time and RT-PCR methods are found to be extremely sensitive, the clinical interpretation of a positive result is considered to be difficult [1,9]. To our knowledge, none of the published reports have analyzed control specimens by RT-PCR to exclude false-positive results and evaluate viral RNA detection shedding in immunocompromised adult patients during symptom free episodes. We did not find any clinically false-positive result for RSV in the specimens taken at set symptom-free moments.

In conclusion, we have shown that Taqman real-time PCR is a sensible and sensitive method for the rapid diagnosis of RSV infection in immunocompromised adults that can be easily implemented in a routine diagnostic setting. It poses a significant improvement over existing virus detection methods for this patient group at risk for serious RSV infection.

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Chapter 6

Frequent detection of human coronaviruses in clinical specimens of patients with respiratory tract infection using a novel real-time RT-PCR

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ABSTRACT

Over the past years human coronaviruses (HCoVs) are increasingly identified as pathogens associated with more severe respiratory tract infection (RTI). Diagnostic tests for HCoVs are not frequently used in the routine setting. It is likely that as a result, the precise role of HCoVs in RTI's is much underestimated. We describe a rapid, sensitive and highly specific quantitative real-time RT-PCR for the detection of HCoV, which can easily be implemented in routine diagnostics. . HCoV was detected in 11% of the 261 clinical specimens from patients presenting with symptoms of RTI, ranging from common cold to severe pneumonia. Only 0.4% of the 243 control specimens obtained from patients without signs of RTI showed the presence of HCoV.

We conclude that HCoVs can be frequently detected in patients presenting with RTI. Real-time RT-PCR provides a tool for large scale epidemiological studies to further clarify the role of coronavirus infection in humans.

INTRODUCTION

Coronaviruses are enveloped RNA viruses that can cause disease in human beings and animals. The human coronaviruses (HCoVs), were first identified in 1962. They belong the the family of *Coronaviridae*, genus coronavirus, and the two human strains, HCoV 229E and OC43, are divided into two antigenic groups. HCoVs are recognized as the second most frequent cause of the common cold syndrome [8]. Over the past years HCoVs are more often held responsible for severe upper and lower respiratory tract infection. They have occasionally been pointed out as a cause of pneumonia in older adults, infants and immunocompromised patients [3,6,9,20]. Also in otherwise healthy adults clusters of infections have been reported as a cause of pneumonia for example in military recruits [21]. Moreover, in a recent outbreak of HCoV in Normandy, the clinical manifestation ranged from mild symptoms to pneumonia [17]. Recently, a heightened interest was documented for the coronavirus because a previously unknown type that does not resemble the known human coronaviruses is being held responsible for the outbreaks of severe acute respiratory syndrome (SARS) in Hong Kong and Toronto [4,5,15,16]. These studies indicate that coronaviruses are more and more identified as a pathogen causing severe respiratory illnesses and that there is a need for reliable and rapid identification of coronaviruses.

The diagnosis of HCoV infections is in part hampered by the difficulty to replicate in cell cultures, whereas serology is sensitive but late and therefore has little clinical significance. As a consequence, efforts have been made to develop more sensitive molecular detection methods such as reverse transcriptase (RT)-PCR and nested RT-PCR [11,18]. These methods have been shown to be very valuable for the determination of the presence of HCoV in different patient populations, such as children with otitis media, patients with multiple sclerosis, immunocompromised patients with pneumonia and frail elderly with symptoms of respiratory tract infection (RTI) [1,4-6,14,20].

Although they are highly sensitive and specific, the current RT-PCR or nested RT-PCR methods are less suitable for routine laboratory detection because they are prone to contamination and still require time-consuming sample handling and post-PCR analysis.

Here we describe the detection of HCoV in a variety of clinical specimens derived from patients presenting with respiratory tract illnesses ranging from common cold to severe pneumonia using a novel highly sensitive and specific Taqman based real-time PCR. In addition, we tested a multiplex format real-time RT-PCR assay for the detection of HCoVs and the novel coronavirus that has been identified in patients with SARS.

MATERIAL AND METHODS

Virus stocks and viral culture. HCoV 229E and OC43 were kindly provided by the Laboratory for Virology, National Institute for Public Health and the Environment (RIVM, Bilthoven, The Netherlands) and propagated on two human embryonic lung cell lines (MRC5 and HEL). Cells and supernatants were harvested after 24, 48 and 72 hours respectively and frozen at –70ºC. Following RNA extraction of each stock, ten-fold serial dilution series were used to determine by an in-house nested PCR which propagated stock contained the most viral particles. The stocks, one of each strain, that contained the most viral particles were used for further experiments to evaluate the real-time Taqman based PCR.

A panel of various respiratory viruses including influenza virus A/PR/8/34, influenza virus B/Lee/40, parainfluenza viruses 1-4 (American Type Culture Collection (Rockville, MD, USA)) and reference strains of rhinovirus 1A, rhinovirus 14, rhinovirus 16, echovirus 12, coxsackie virus A9, RSVA Long strain, RSV B 9320 and SARS associated coronavirus were used to determine the specificity of the real-time Taqman based PCR.

Clinical specimens. Clinical specimens were received at the hospital virology laboratory and consisted of : (i) Nasal washes (NW) and combined nose and throat swabs (NTS) from patients presenting with upper- or lower respiratory symptoms, (ii) bronchoalveolair lavages (BAL) and NTS that were obtained from adult patients admitted at the hospital with pneumonia. NTS samples from healthy volunteers and NTS that were collected at set time-points from patients without signs of respiratory tract infection who participated in a prospective six month follow-up study to assess the role of respiratory viruses following bone marrow transplantation were used as control specimens. Each sample was transported in 5 ml of virus transport medium. Nasal wash specimens, NTS and BAL were vortexed for 10 s and centrifuged at 2,000 x g for 15 min. One ml of the supernatant was used directly for routine virus culture of other respiratory viruses (influenza viruses, respiratory syncytial virus, parainfluenza viruses, picornaviruses and adenovirus). The remaining material was stored at –70ºC until further processing.

Viral RNA isolation and cDNA synthesis. RNA extraction was performed using the MagnaPure LC Total Nucleic Acid Kit (Roche Diagnostics, Mannheim, Germany) as described previously [13]. The RNA was then eluted either in 100 µl 40 ng/µl polyA RNA before performing a one-tube reverse transcription (RT)-PCR or eluted in 100 µl elution buffer and directly used for cDNA synthesis. The reverse transcription and cDNA synthesis were both performed as published previously [19] and the products were stored at -70°C until further use.

In-house nested PCR. An in-house nested PCR was carried out for HCV OC43 and 229E. First-round amplification primers and nested primers were derived from literature, targeting the nucleocapsid (N) gene, with

one minor modification: in contrast to the published sequence we omitted an excess T on position 13 from the nested anti-sense primer [11]. A one-tube RT-PCR followed by a second (nested) amplification was applied as described previously [13] using a PE 9600 Thermocycler (Perkin Elmer). PCR products were visualized on an ethidium bromide-stained agarose gel using UV illumination. A 5 µl 100 base-pair marker was used, to control fragment lengths.

Table 1. Selected primers and probes for the real-time RT-PCR of HCoV-229E and HCoV-OC43

HCoV type(target)	Primer/probe	Sequence	Nucleotide position[a]
229E (N gene)	N229E-1	5' CAGTCAAATGGGCTGATGCA	154-173
	N229E -2	5' AAAGGGCTATAAAGAGAATAAGGTATTCT	230-201
	N229E-p	5' CCCTGACGACCACGTTGTGGTTCA	199-176
OC43 (N gene)	NOC43-1	5' CGATGAGGCTATTCCGACTAGGT	577-599
	NOC43-2	5' CCTTCCTGAGCCTTCAATATAGTAACC	652-626
	NOC43-p	5' TCCGCCTGGCACGGTACTCCCT	601-622

[a] Primer and probe positions are given according to their position on the nucleocapsid gene

Real-time Taqman PCR. Type specific primers and probes for HCoV OC43 and 229E were selected using primer express software (PE Applied Biosystems) and were based on the genomic regions of high conservation of the N-gene. The forward and reverse primers (N229E-1, N229E-2, NOC43-1 and NOC43-2) and probes (N229E-p and NOC43-p) that were used are shown in Table 1. The primers and probes of HCoV OC43 and 229E were tested for possible interactions to make sure they could be used in combination. After optimization of the primer and probe concentrations, samples were assayed in duplicate in a 25 ul reaction mixture containing 5 ul of cDNA, 12.5 ul of 2xTaqMan Universal PCR Master Mix (PE Applied Biosystems), 150 nM and 450 nM for HCoV 229E and OC43 forward primers respectively, 150 nM and 450 nM of the HCoV 229E and OC43 reverse primers respectively, 50 nM of the HCoV 229E probe and 100 nM of the HCoV OC43 probe. The fluorogenic probes that can be labeled with different fluorogenic dyes were both labeled with the 5' reporter dye 6-carboxy-fluorescein (FAM) and a 3' quencher dye 6-carboxy-tetramethyl-rhodamine (TAMRA). Amplification and detection were performed with the ABI Prism 7700 sequence detection system under the following conditions: 2 min at 50ºC to require optimal AmpErase UNG activity, 10 min at 95ºC to activate AmpliTaq Gold DNA polymerase followed by 45 cycles of 15 s at 95ºC and 1 min at 60ºC. The primers and probe for the SARS associated coronavirus were used targeting the polymerase gene as published recently [2].The primers and probes of HCoV OC43, 229E and the SARS associated coronavirus were tested for possible interactions to make sure they could be used in combination in a multiplex assay.

Virus quantification. To estimate the quantity of the virus load, viral particles were expressed as relative units (RU). Above a threshold cycle of 36 the quantitive value of RNA copies can no longer be considered accurate. Therefore every value above threshold cycle 36 and below the detection limit threshold cycle 45 was assumed 2. Every amplification cycle represents a 2-fold increase in viral RNA copies. The viral load was

expressed as 2-fold increase per cycle relative to a baseline value of 2 copies at threshold cycle 36 (RU= $2^{36-\text{threshold cycle}}$).

RESULTS

Sensitivity and specificity. Limiting dilutions series showed similar sensitivity of the in house nested PCR and the real-time RT-PCR for HCoV. To compare the sensitivity of the in-house nested PCR with the real-time RT-PCR for HcoV on clinical samples a total of 86 NW specimens derived from asthmatic and otherwise healthy subjects with upper and/or lower respiratory tract symptoms were analyzed for HCoV by both the in-house nested PCR and the real-time RT-PCR. As shown in table 2. 14/86 were found positive by real-time RT-PCR compared to 10/86 by the in house nested PCR. The real-time RT-PCR performed better in the specimens containing a low viral load compared to the nested PCR (Figure 1.).

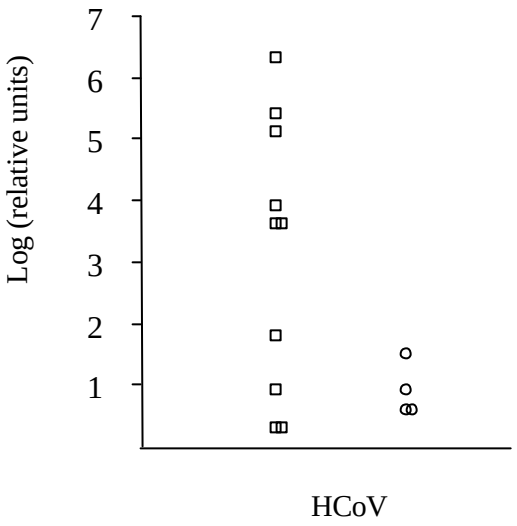

Figure 1. Virus quantity expressed as relative units (RU) of HCoV that could be detected in the clinical specimens (n=14). The round symbols (○) represent the clinical samples detected by real-time Taqman PCR only, whereas the square symbols (□) represent the clinical samples detected by both the in-house nested PCR and the real-time Taqman PCR.

The real-time RT-PCR for HCoV was highly specific: none of the other respiratory viruses (rhinovirus 1A, 14 and 16, respiratory syncytial virus A Long strain and B 9320, parainfluenza viruses 1-4, influenza virus B/Lee/40, influenza virus A/PR/8/34, coxsackievirus A9, echovirus 12 and the SARS associated coronavirus) revealed a positive signal in the real-time RT-PCR assay. To evaluate the possibility of clinically false-positive results, NTS were taken from 30 asymptomatic subjects during the winter season. In none of the NTS HCoV was detected by real-time RT-PCR (table 2).

The real-time PCR for HCoV could successfully be combined with a real-time PCR for SARS associated coronavirus. Limiting dilution series using a single (primers and probe for the SARS associated coronavirus only) and multiplex format (combination of primers and probes for the HCoV and SARS associated coronavirus) showed similar sensitivity in the detection of SARS associated coronavirus RNA and HCoVs.

Table 2. Detection of HCoV by real-time RT-PCR and/or nested RT-PCR in clinical specimens

	n=	**Nested RT-PCR** (%)	**Real-time RT-PCR** (%)
NW			
URT/LRTI*	86	10 (11.6)	14 (16.3)
NTS			
URTI/LRTI*	151	ND	10 (6.6)
Pneumonia	13	ND	2 (15.4)
Asymptomatic bone marrow transplant receipients	243	ND	1 (0.4)
Healthy controls	30	0	0
BAL			
Pneumonia	11	ND	2 (18.2)

NW= nasal washes, **NTS**=combined nose and throatswabs, **BAL**= bronchoalveolair lavage
* URTI/LRTI= patients presenting with symptoms of upper and/or lower respiratory tract infection

Detection in clinical specimens and control specimens. To evaluate the real-time RT-PCR assay for HCoV we analyzed a total of 261 clinical specimens received at the hospital virology laboratory: (i) 86 NW specimens and 151 NTS were obtained from patients presenting with symptoms of upper and/or lower respiratory tract infection and (ii) 11 BAL specimens and 13 NTS derived from patients admitted to the hospital with pneumonia. Moreover, 243 control NTS were evaluated from bone marrow transplant receipients without signs of respiratory tract illness. In total 28/261 (11 %) of the clinical specimens revealed HCoV. Human coronavirus was detected in the BAL of 2/11 (18.2%) patients. In addition, 2/13 (15.4%) NTS from patients admitted at the hospital with pneumonia

revealed HCoV (table 2). In contrast, HCoV RNA was only detected in 1/ 243 (0.4%) NTS that were taken at set time points without obvious signs of respiratory illness (table 2).

Five patients with upper respiratory tract symptoms and a positive real-time RT- PCR for coronavirus were followed during their infections. A total of 24 nasal washes were obtained just after the presentation of common cold symptoms up to 60 days. The virus load was expressed as RU (RU= $2^{36\text{-threshold cycle}}$). As shown in figure 2, we were able to detect and quantify corona virus in nasal washes up to 7 days after the initial presentation of common cold symptoms in 3 patients, and up to 14 days in 1 patient. In 1 patient (patient 5) the viral load was below the level of reliable quantitation.

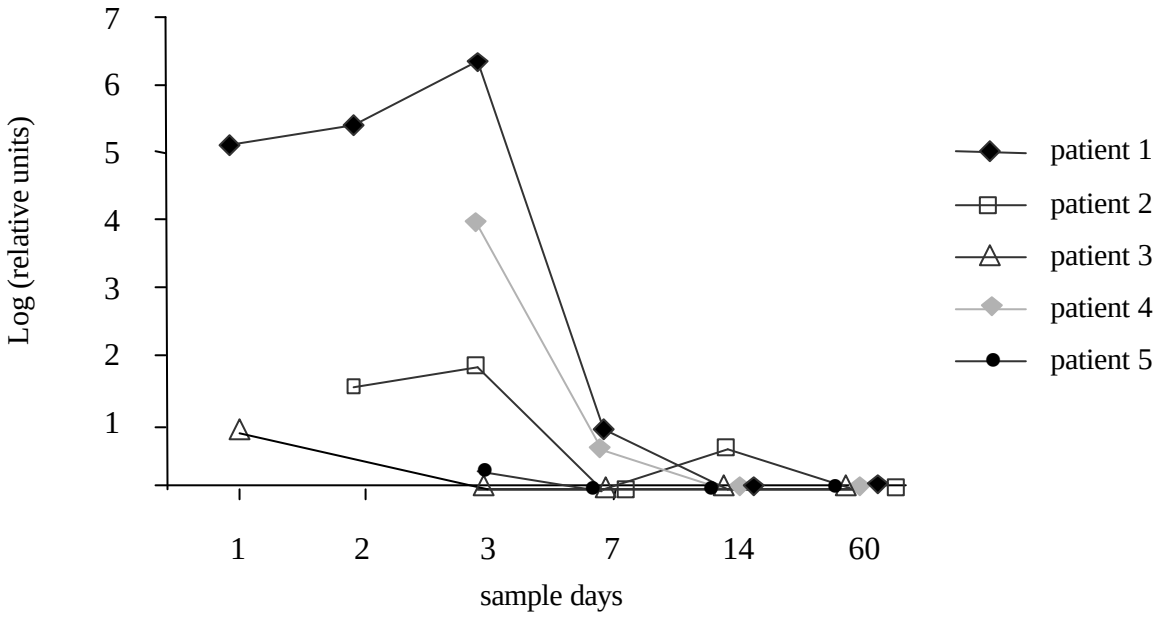

Figure 2. Longitudinal follow-up of 5 patients with either OC43 or 229E coronavirus infection. Quantitative analysis was performed using the multiplex Taqman PCR. The quantity is expressed on the y-axis as relative units (RU). RU= $2^{36\text{-threshold cycle}}$

DISCUSSION

Our findings demonstrate, that HCoV is frequently detected in clinical specimens received at the virology laboratory from patients presenting with respiratory tract infection . The novel real-time PCR assay allows rapid and specific detection of HCoVs in patients with various presentations of respiratory tract infection.

Since increasing evidence exists that either the known or newly identified human coronaviruses might be involved in more severe disease there is a need for more rapid and reliable diagnosis. At present, a great deal of attention is drawn towards patients with severe acute respiratory syndrome (SARS). A novel coronavirus that has been identified in the majority of the patients is the primary cause of SARS

[2,7,10,15,16]. Genetic characterization of this novel coronavirus shows considerable differences from human coronaviruses 229E and OC43 [2]. The here described real-time RT-PCR can detect the novel SARS-associated coronavirus, when used in a multiplex format. However, it has yet to be determined whether this is a favorable format since the clinical presentation of SARS differs from the assumed clinical presentation of HCoV infection. However, for example advanced age and underlying disease have also been associated with a more severe presentation of HCoV infection [4,5]. Also, in case reports HCoV has been associated with pneumonia following autologous bone marrow transplantation and we recently identified HCoV using nested PCR in the broncho-alveolair lavage of a severely immunocompromised patient with pneumonia [6,20]. Interestingly, in the present study we detected HCoV by real-time RT-PCR in the BAL of two patients presenting with severe pneumonia, and in the NTS from patients admitted to the hospital with pneumonia, which again suggests that HCoV may be the cause of severe disease in some patients.

With the use of molecular detection methods Nicholson et al. already showed that 26% of upper respiratory tract infections in elderly people living at home were due to HCoVs and the identification of a recent community outbreak of HCoV OC43 in France was facilitated with the use of RT-PCR [4,5,12,15-17]. Although valuable in a research setting these methods are less suitable for routine laboratory detection because they still require time-consuming sample handling and post-PCR analysis and are consequently prone to contamination. Besides being rapid, real-time RT-PCR assay has the advantage of a standardized protocol that can easily be applied for other respiratory viruses: the RT-PCR can be performed under uniform amplification conditions, thereby using target specific primer and probe sets.

Another deficit is that most studies using RT-PCR for the detection of HCoV lack proper control groups to evaluate the clinical value of a positive RT-PCR. To gain insight into the relevance of a positive assay we followed five symptomatic patients during the coronavirus infection and also took samples from asymptomatic individuals. The follow-up of the five symptomatic patients showed that HCoV RNA could be detected up to 14 days after infection by real-time RT-PCR. Moreover, we tested specimens derived from patients without obvious signs and symptoms of respiratory infection. None of the samples derived from healthy individuals contained corona viral RNA. At one timepoint, just after the bone marrow transplantation, we detected HCoV in a NTS from a bone marrow transplant recipient without obvious upper and/or lower respiratory tract infection. It might well be that the patient was suffering from a minor cold but that these symptoms remained unnoticed. From these results we conclude that a HCoV positive finding by real-time RT-PCR in a symptomatic patient has diagnostic significance.

Diagnostic tests for HCoVs are not frequently used in the routine setting. Serological methods do not allow rapid virus identification and although both HCoV OC43 and 229E can be propagated on specialized cells, the approach lacks sensitivity, is time-consuming and often requires specialist expertise. In addition, virus isolation is often considered redundant and without clinical consequence

as HCoVs are thought to be mainly associated with the common cold syndrome. It is likely that as a result, the precise role of coronaviruses in respiratory infections is much underestimated because of the lack of practical diagnostic tools.

We realize that the specimens analyzed in the present study, received at the virology laboratory, probably represent a selected group of patients in which a respiratory virus is considered as a possible pathogen on clinical grounds. The results however indicate that HCoV is frequently detected and that the novel real-time PCR assay provides a tool for large scale epidemiological studies to further clarify the role of coronavirus infection in humans.

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Chapter 7

Polymerase chain reaction is more sensitive than viral culture and antigen testing for the detection of respiratory viruses in adults with hematological cancer and pneumonia

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ABSTRACT

We retrospectively analyzed the value of polymerase chain reaction (PCR) for the detection of respiratory viral infections in 43 patients with hematologic cancer whose bronchoalveolar lavage (BAL) samples had been stored. In addition, 17 nose-throat (NT) swabs and 29 blood samples had been obtained. PCR was performed to detect parainfluenza viruses 1-3, respiratory syncytial virus, rhinovirus, influenza viruses A and B, enteroviruses and coronaviruses. Virus cultures or antigen testing of BAL samples revealed 9 respiratory viruses in 8 patients. By use of PCR, 8 more respiratory viruses were detected in another 7 patients, increasing the rate of identification from 19 % to 35 % ($P<0.0005$). Available NT swabs yielded the same results with PCR as did BAL samples. We conclude that PCR is more sensitive than viral culture or antigen or serological testing for detection of respiratory viruses in patients with hematological malignancies, and that it offers the possibility for early, more rapid diagnosis.

INTRODUCTION

Pneumonia is one of the most common infectious complications of stem cell transplantation (SCT) and cytotoxic treatment for hematological malignancies. Traditionally, pulmonary infections in patients who undergo SCT or who receive cytotoxic agents have been mostly attributed to bacteria, fungi, and herpesviruses. During the past decade, respiratory viruses have increasingly been recognized as important causes of severe lower respiratory disease in these patients [8,16,30,32,35]. Respiratory syncytial virus (RSV), influenza viruses, parainfluenza viruses, adenoviruses, and picornaviruses have all been identified as significant pathogens of community-acquired and nosocomial infection.

At present, viral culture is the “gold standard” for laboratory diagnosis of respiratory virus infections. However, it is not suitable as a rapid diagnostic test, because culture usually takes 2-10 days to yield results, and, therefore, its clinical value is limited. To overcome these limitations, more rapid diagnostic techniques, such as direct viral antigen detection, have been introduced in the routine laboratory setting. These techniques provide results faster, but they are generally considered to be less sensitive and specific than is conventional cell culture. Also, they are not suitable for detection of all respiratory viruses; for example, antigen testing for rhinoviruses is not possible, because too many subtypes exist and co-circulate at the same time [5,26]. Although it has been studied in several patient groups, the role of respiratory virus infections as the cause of severe pulmonary complication in patients receiving cytoreductive therapy or undergoing SCT is not yet clarified and may be underestimated in previous studies, particularly in those studies that have relied on virus culture.

PCR, either in single or multiplex format, has proven to be an extremely specific and sensitive method for the detection of respiratory viruses [22,28]. In our hospital, nested

reversed transcriptase-PCR (RT-PCR) techniques have been developed to detect the following respiratory viruses: parainfluenza viruses 1-3, RSV, rhinoviruses, influenza viruses A and B[28], enteroviruses and coronaviruses.

In this study, we investigated the value of PCR for the detection of respiratory viral infections in 43 adults with hematological cancer who also had signs of pneumonia to further establish the role of respiratory viruses in these patients.

PATIENTS AND METHODS

Patients. The University Medical Center at Utrecht, the Netherlands is a referral center for treatment of hematological malignancies in adults. Every year, approximately 75 patients undergo either autologous or allogeneic SCT.

From October 1997 through May 2000, all patients from the hematology ward and the hematology outpatient clinic who underwent bronchoalveolar lavage (BAL) were selected for study through the database of the Department of Virology. Since October 1997, BAL samples obtained from patients with hematological malignancies have been routinely stored at the hospital's diagnostic virology laboratory. For this retrospective study, 43 adult hematological cancer patients who also had signs of pneumonia and radiographic pulmonary abnormalities and whose BAL samples had been stored were considered assesable. We reviewed the patients' charts to obtain the following information: underlying disease and therapy; antimicrobial treatment; additional bacterial, fungal, and viral culture data or antigen testing results; serological data; clinical features, and outcome. A total of 43 BAL specimens from these patients had been investigated routinely for the following pathogens: bacteria, mycobacteria, fungi, herpesviruses, and respiratory viruses (influenza viruses, RSV, parainfluenza viruses, picornaviruses and adenoviruses). Nose-throat (NT) swabs had also been obtained from 17 of these 43 patients within one week of the BAL sample. These NT swabs had also been stored after conventional testing for respiratory viruses. In addition, paired serum samples had been obtained from 29 patients for detection of atypical bacterial (e.g., *Mycoplasma pneumoniae*, *Chlamydia* species, *Legionella* species) and respiratory virus pathogens.

The stored BAL samples and NT swabs were subsequentely analyzed by use of PCR techniques for the detection of respiratory viruses.

SCT regimens, infection prophylaxis and infection-prevention measures. Patients with an expected duration of neutropenia of >7 days received antibacterial prophylaxis with oral administered ciprofloxacin (500 mg twice per day) and orally administered antifungal prophylaxis with amphotericin B tablets (200 mg 4 times per day) and fluconazole (50 mg once per day). The antimicrobial regimen was continued until the granulocyte count had increased to $> 0.5 \times 10^9$/L. For prevention of bacteremia caused by α-hemolytic streptococci, patients received clindamycin (300 mg 3 times per day) while they had neutropenia in case of high-dose cytarabine ($\geq$ 500 mg/m^2). Patients undergoing SCT received intravenously cephalothin (1 g 6 times per day) after transplant while they had neutropenia. Patients who underwent allogeneic SCT routinely received valacyclovir (500 mg twice per day) and cotrimoxazole (480 mg once per day) during the first 12 months after transplantation. In addition, patients who had a positive result of a cytomegalovirus pp65 test during

the first 3 months after they underwent allogeneic SCT received preemptive therapy with ganciclovir [29]. Hospitalized patients were cared for in a single rooms with free entry for staff and visitors. Careful handwashing and the use of low-microbial-count food were the only preventive measures used for these patients. Pulmonary infections were considered to be hospital acquired if symptoms developed $\geq$ 4 days after admission.

Diagnostic methods for the routine detection of respiratory viral pathogens. Nasopharygeal and throat swabs, which were placed in the same viral transport media, and BAL samples which were placed in a tube containing virus transport medium, were taken for viral culture; they were either transported to the laboratory immediately or stored at 4°C for a maximum of 24 hours. The material was divided: some of it was frozen and stored at -70°C for further analysis by PCR, and some was directly used for virus culture. These cultures were performed by inoculating HEp-2C, R-HELA, and tertiary monkey kidney (t-MK) cells with 100 µl of each clinical sample for the detection of respiratory viruses (adenoviruses, parainfluenza viruses, RSV, influenza viruses and picornaviruses). The cultures were examined for cytopathic effect twice a week for 10 days. In positive cultures, virus was identified by immunofluorescence with commercial monoclonal antibodies (Dako Imagen, Uithoorn, the Netherlands) for influenza A and B viruses, RSV, parainfluenza viruses 1-3 and adenoviruses. Rhinoviruses were distinguished from enteroviruses by means of acid-lability testing.

Rapid antigen testing was performed after 1-2 days of culture, usually before a cytopathic effect could be noticed. Immunofluorescence microscopy that used virus-specific monoclonal antibodies (Dako Imagen) was used to detect RSV, parainfluenzaviruses 1-3, influenza A and B viruses and adenoviruses.

Only paired serum samples were used for serological detection of respiratory viral illness, and a positive diagnosis was defined as a four-fold rise in virus specific antibody titers. The standard serologic test complement fixation was used for RSV, influenza A and B, parainfluenza virus 1-3, and adenovirus infection. In addition, the indirect immunofluorescence assay was used for RSV and influenza A and B.

RNA extraction from clinical specimens and nested PCR. PCR was performed to detect influenza A and B virus, parainfluenza virus 1-3, picornaviruses (rhinovirus and enterovirus), RSV and coronaviruses on the stored BAL samples obtained from all 43 patients and on the NT swabs obtained from 17 patients ;the NT swabs had been obtained within 1 week of the BAL.

Primers were obtained from literature or selected from GenBank on conserved regions of the genes of the matrix protein for influenza A virus, of the haemagglutinin gene for influenza B virus[28], the 5' noncoding region for the picornaviruses [2], the nucleocapsid protein for RSV A and B, the haemagglutinin-neuraminidase glycoprotein for parainfluenza 1,2,3 [7], and the nucleocapsid protein for coronavirus 229E and OC43 [19]. Nucleic acid extraction was performed from 100 µl of patient material in accordance with the method of Boom et al.[3]. For all PCR reactions a one-tube RT-PCR was followed by a nested-PCR, essentially as described by Nijhuis et al.[21]. Modifications of this method consisted of optimization of each separate PCR reaction by serial dilution of $MgCl_2$ and primer concentrations. PCR was performed on a PE 9600 Thermocycler (ABI). Rhinoviruses were identified by Bgl I digestion of the picornavirus RT-PCR amplicons [23]. PCR products were visualized on a ethidium bromide-stained agarose gel by use of ultraviolet illumination.

Table 1. Characteristics and outcomes of 43 adult hematological cancer patients with chest radiographic abnormalities.

Parameter	Total patients n=43	Patients with respiratory virus (n=15)	Patients without respiratory virus (n=28)
Age (median, range)	46 (17-66)	45 (18-65)	43 (17-66)
Sex (M/F)*	28/15	8/7	20/8
Underlying disease			
Acute myelogenous leukemia	10	4	6
Acute lymphoblastic leukemia	6	3	3
Chronic myelogenous leukemia	6	-	6
Multiple myeloma	7	4	3
Non Hodgkin lymphoma	6	2	4
Myelodysplastic syndrome	4	1	3
Other	4	1	3
Treatment			
Stem cell transplantation*	28	11(73%)	17 (61%)
Allogeneic	24	9	15
Autologous	4	2	2
Cytotoxic therapy	15	4 (27%)	11 (39%)
Granulocytopenia < 0.5 x 10^9/L*	18	5 (33%)	13 (46%)
Patients using immunosuppressives*	24	9 (60%)	15 (54%)
Signs and symptoms*			
Fever	30	10 (67%)	20 (71%)
Cough	28	12 (80%)	16 (57%)
Dyspnea	23	8 (53%)	15 (54%)
Malaise	18	8 (53%)	10 (29%)
Rhinitis	3	-	3 (11%)
Pharyngitis	1	1 (7%)	-
Type of specimen			
BAL fluid	43	15	28
BAL fluid and nose-throat swab	17	7	10
Nosocomial /community-acquired respiratory disease*	10/33	3/12	7/21
Median time between transplantation and pulmonary abnormalities (months)*	4	5	3
During 'Winter' season (October-March)*	26	11 (73%)	15 (54%)

* None of the differences between the groups were significant

Statistics. Descriptive statistics were expressed as median values. χ^2 Analysis was performed to determine the degree of significance between the various variables.

RESULTS

Patient characteristics. The demographic characteristics, underlying disease, conditioning therapy, use of prophylaxis, and immunological status of the patients are shown in Table 1. The majority of patients presented with signs and symptoms of respiratory disease. Fever (in 30 [70%] of 43 patients), cough (in 28 [65%]) and shortness of breath (in 23 [53%]) were the most common complaints. Ten (23%) of 43 patients developed signs and symptoms of pneumonia at the time of hospital admission, and 33 patients (77%) developed community-acquired pneumonia. Twenty-eight (65%) of 43 patients had undergone SCT; the median duration from transplantation until the onset symptoms of respiratory disease was 4 months (range, 0-28 months). Twenty-six (60%) of 43 patients developed pneumonia during the winter season (October-March).

Table 2. Detection of respiratory viruses by culture and/or antigentesting and PCR in either BAL or NT-swabs and paired serum samples.

virus	Material				
	BAL (No. of patients=43)		NT-swabs (No. of patients=17)		Paired sera (No. of patients=29)
	Method				
	Culture and/or antigentesting	PCR	Culture and/or antigentesting	PCR	Serology
Respiratory Syncytial virus	4	5	4	4	4
Human Rhinovirus	3	5	0	0	ND
Para influenza viruses 1,2,3	0	2	0	0	0
Human Coronaviruses	0	1	0	0	ND
Influenza viruses A,B	2	2	1	2	0
Enteroviruses	0	2	0	1	ND
Adenoviruses	0	ND	0	ND	3
Total	9	17	5	7	7

ND= not done

Detection of respiratory viruses. By means of culture, antigen testing, or both, 9 respiratory viruses were identified in 8 patient, of which 4 were RSVs, 3 were rhinoviruses, and 2 were influenza A viruses. The same 9 respiratory viruses were detected by the nested RT-PCR. One of the patients had an infection with a respiratory virus twice. Initially, this patient was admitted with pneumonia caused by RSV, which subsided spontaneously within

10 days. Then, the patient, who was still an inpatient at the hospital, developed nosocomial pneumonia again 1 week later, which was caused by culture-proven influenza A. An additional 8 respiratory viruses were detected by PCR in another 7 patients (Table 2).

One patient had a dual infection with rhinovirus and parainfluenza 1 virus. In total, 17 respiratory viruses were detected by PCR in 15 (35%) of 43 patients, compared with 9 respiratory viruses (19%) in 8 patients detected by culture, antigen testing, or both (P<.0005). Paired serum samples were available for 29 patients. Serologic testing showed a 4-fold increase in RSV-specific IgG antibody titer in only 4 patients and a 4-fold increase in titer for adenovirus in only 3 patients.

A combined NT-swab was obtained from 17 of 43 patients within 1 week of the BAL sample. In 7 patients with respiratory virus disease from whom samples of both NT and BAL were available, the nested RT-PCR on NT samples always yielded the same results as the BAL samples (Table 2).

Table 3. Causes of pulmonary abnormalities in 43 adult patients with hematological malignancies

Pathogens/ Other causes of radiographic abnormalities	n=43
Bacteria	4
Bacteria and respiratory virus	2
Respiratory virus	9
Respiratory virus plus fungi (proven/probable)	2 (1/1)
Fungi (proven/probable)	9 (4/5)
Other*	5
Other plus virus	2
Unknown	10

* Other causes: bronchiolitis obliterans (n=2), Epstein-Barr virus associated post transplantation lymfoproliferative disease (n=2), acute toxic lung injury (n=1), and CMV pneumonia (n=1).

Other causes of pneumonia. In 10 patients (23%), no cause of pneumonia was found (Table 3). Respiratory virus pathogens could be detected in 15 patients (35%). In 6 (40%) of 15 patients in whom a respiratory virus pathogen was detected, another cause of pulmonary infection or lung injury was clinically probable. Two of these patients were thought to have

pneumonia caused by both a bacterium and a respiratory virus (*Staphylococcus aureus* and rhinovirus in one patient, and *Haemophilus influenzae* and rhinovirus in the other); of another 2 patients, 1 had a proven (*Aspergillus fumigatus*) and 1 had a probable pulmonary fungal infection together with an infection with enterovirus and influenza A virus, respectively. Another patient had a posttransplantation lymphoproliferative disease with pulmonary involvement after receiving a stem cell transplant from a matched unrelated donor, in combination with an enterovirus infection. In 1 patient, coronavirus was detected in addition to a bronchiolitis obliterans. Four (9%) of 43 patients had pneumonia probably caused by 1 (in 3 patients) or 2 (in 1 patient) bacteria. *Enterobacter* species, *Pseudomonas* species, *H. influenzae* and *Stenotrophomonas maltophilia* were isolated from these patients. A total of 9 patients (21%) had a proven (in 4 patients) or probable (in 5) pulmonary infection with fungi. Five patients had other causes of pulmonary disease. One patient had a progressive Epstein-Barr virus-associated posttransplantation lympfoproliferative disease with pulmonary involvement after receiving a stem cell transplant from a matched unrelated donor; 2 patients had bronchiolitis obliterans; another patient had a CMV pneumonitis; and 1 patient developed toxic lung injury after transplantation.

Treatment. Two of the 5 patients with RSV pneumonia were treated with aerosolized ribavirin (2 g 3 times per day, for a minimum of 7 days). At the start of ribavarin treatment, these 2 patients had had symptoms of upper respiratory tract infection for 1 week. Both patients recovered completely after 1 week of treatment. Another 2 patients also recovered from RSV pneumonia, but without administration of ribavirin. One patient died. This patient had contacted RSV pneumonia during a recurrence of acute myelogenous leukemia shortly after receiving an allogeneic stem cell transplant from a matched unrelated donor; also, the patient did not receive treatment, because the diagnosis was made by a positive PCR for RSV only after death.

Comparison between patients with pneumonia with or without respiratory virus. Patients with pneumonia caused by a respiratory virus were compared with patients who had pneumonia that was not caused by a respiratory virus with regard to the following characteristics : underlying disease, treatment, immune status, use of immunosuppressives, signs and symptoms, type of specimen obtained, presence of nosocomial or community-acquired respiratory disease, time of transplantation, and the period of the year that they acquired their infection (Table 1). There was no significant difference in parameters between the 2 groups, although there seems a tendency toward more male patients, use of immunosuppressives and the presence of neutropenia in the group of patients who had pneumonia that was not caused by a respiratory virus. The majority (11 [73 %] of 15) of the cases of respiratory virus-associated pneumonia occurred during the winter season (October-March), whereas the occurrence of pneumonia without detection of respiratory virus was

spread equally throughout the year (15 [54%] of 28 cases during the winter months vs. 13 [46%] of 28 cases during the summer months).

DISCUSSION

For 43 patients with hematological cancer and pneumonia, stored BAL samples yielded significantly more respiratory viruses when a nested RT-PCR was performed as compared with standard culture, rapid culture, or both. Serologic testing was only of value in 4 cases of acute RSV infection and in 3 cases of adenovirus infection. These results indicate that previous studies relying on virus culture, antigen testing, or both to determine the incidence and role of respiratory viruses in this patient group may have underestimated the true incidence [8,16,30,32,35].

During the past decade, respiratory viruses have been increasingly recognized as causative agents of respiratory tract infections in severely immunocompromised patients [8,16,30,32,35]. High frequencies of nosocomial acquisition, persistence of infection beyond the time periods reported for immunocompetent patients and a high frequency of pneumonia and death have been found in association with respiratory viral infections in immunocompromised patients [35]. As in some other studies, by PCR, we found a relatively high incidence of respiratory virus-associated pneumonia in immunocompromised patients. Reported incidences of respiratory virus infections were 26-36% in adult bone marrow transplant recipients with acute upper and lower respiratory illnesses; for immunocompromised patients, the rate was 19% [4,18,32]. However, we cannot confirm some of the reported high frequencies of nosocomial acquisition, nor did we find a high rate of deaths due to respiratory virus-associated pneumonia.

Overall, in studies published elsewhere, RSV accounted for the majority of respiratory virus infections with high mortality rates [4,33,35]. RSV-related mortality rates as high as 83% have been reported in hospitalized adult patients with leukemia, and the rates have been as high as 78% in persons who undergo SCT[33] . Prompt therapy of RSV infections with aerosolized ribavarin with or without intravenous immunoglobulin appear to impact favorably on the frequency of progression to pneumonia and death in some studies, but randomized controlled studies are lacking [11,27]. Data from our study are consistent with those of previous studies that have shown that RSV is the most prevalent respiratory virus in persons with respiratory virus-associated pneumonia. The mortality rate in our study, however, was only 20%.

Influenza and parainfluenzaviruses have also been reported frequently in immunocompromised patients during community-outbreak periods of these respiratory viruses [15,17,31,34]. In particular, parainfluenza virus infection may be an important cause of life-threatening pneumonia in patients who undergo SCT or who have received treatment

for leukemia, with mortality rates of up to 66 % [15,31]. The incidence and severity of pneumonia in immunocompromised patients caused by influenza virus varied in several studies. In one study [34] influenza was isolated in 29% of persons who underwent SCT and who had an acute respiratory illness, and it had been complicated by pneumonia in 75% of these patients. Other researchers have concluded that influenza A virus in immunocompromised patients only occasionally causes severe complications, and that it is often mild and self-limiting [17]. We found 2 patients with influenza A virus-associated pneumonia and 2 patients with parainfluenza virus infections. None of these patients received antiviral therapy, and all recovered without sequelae.

Recently, rhinoviruses have also been identified as pathogens with the potential to infect the lower respiratory tract. Gosh et al. [10] described 22 cases of rhinovirus-associated infections in myelosuppressed adult blood and bone marrow transplant recipients early after transplant, 7 of whom developed fatal pneumonia. In this study, rhinoviruses were detected by means of conventional methods. Of interest, with the use of molecular diagnostics, we documented an increased involvement of 30% of rhinoviruses in virus-associated pneumonia, indicating that rhinoviruses may play a serious role as a cause of pneumonia in the immunocompromised.

We detected coronavirus in a sample obtained from one of our immunocompromised patients. Our observation is in line with the findings of another study, which demonstrated that pneumonia caused by coronavirus occurred in a patient who had received an autologous bone marrow transplant to treat breast cancer [9]. We also analyzed our samples for the presence of enterovirus, although the lower respiratory tract is not the usual site of enterovirus infection. However, in immunocompromised patients with pneumonia, presence of enterovirus in BAL specimens was demonstrated in several studies [12,13,24]. These results were confirmed in our own study, in which 2 patients revealed the presence of an enterovirus and thereby demonstrated that these infections should be considered as a cause of pneumonia in severely immunocompromised patients.

Although a method for the detection of adenovirus with PCR has been described elsewhere [6], we only detected adenovirus infections by serologic testing. We found a 4-fold increase in serum titer of adenovirus in 3 patients. No adenoviruses were detected by standard culture or antigen detection; a generic PCR for adenovirus infection was not yet available at our laboratory.

There are still some limitations to be considered when PCR is used as diagnostic tool for the detection of respiratory viruses. First, we cannot completely rule out contamination of the BAL sample from the upper respiratory tract. As was stated before, we found 100% concordance between PCR results on BAL and NT samples. Thus, we cannot rule out the possibility of contamination. However, this uncertainty is intrinsically related to the use of bronchoalveolar lavage. Second, we cannot rule out the possibility of positive PCR results

having little clinical significance, since severely immunocompromised patients are known to shed virus for long periods of time [14]. Therefore, the exact meaning of a positive PCR result still needs to be determined in prospective studies.

Because different antiviral agents are now available or under development for treatment of RSV, influenzavirus A and rhinovirus infections [1,20,25], it is be important to improve methods to rapidly diagnose respiratory illness that are caused by respiratory viruses in immunocompromised patients. A rapid and sensitive method for detecting respiratory viruses is essential to implement prompt measures, both to start treatment as soon as possible in patients who are at risk of developing pneumonia caused by respiratory viruses and to prevent or limit nosocomial spread of infection. We have showed that PCR might be an important tool to accomplish this goal.

In conclusion, we have shown that molecular diagnostic techniques significantly increase the detection rate of respiratory viruses in patients with hematological cancer and pneumonia, as compared with traditional methods. However, prospective surveillance studies are still necessary to further establish the clinical value of these techniques.

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Chapter 8

Respiratory viruses are a major cause of respiratory tract disease in adult recipients of stem cell transplantation

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(Submitted)

ABSTRACT

During a 6-month period 72 recipients of autologous and allogeneic stem cell transplantation (SCT) were monitored to determine the incidence and severity of respiratory virus infection and to assess the diagnostic value of real-time polymerase chain reaction (PCR) in the detection of respiratory virus compared to viral culture. In case of upper or lower respiratory tract infection (URTI/LRTI) nose-throat (NT) swabs or broncho-alveolair lavage (BAL) samples were taken for virus culture and quantitative PCR. In addition, NT samples from all SCT recipients were taken at predetermined intervals, in the absence of any clinical symptoms. A total of 52 episodes of respiratory upper (n=41) or lower (n=11) RTI were observed in 40 of 72 patients. RTI could be associated with a respiratory virus in 11 of 52 (21%) episodes by viral culture, but by real time PCR in 33 of 52 episodes (63%, $P<0.0001$). Especially in LRTI, real time PCR was much more sensitive than viral culture (73% versus 9%, $P=0.008$) in the detection of respiratory viruses. Rhinovirus was detected most frequently (19 out of 33 episodes, 58%), predominantly in URTI. Interestingly, we did not observe progression from viral URTI to LRTI in our patients, and in addition, patients presenting with LRTI had experienced no previous complaints of an URTI. In samples obtained one week after start of complaints from patients who had a respiratory virus disease, the initial respiratory virus could still be detected by means of real-time PCR in 16 out of 25 (64%) samples, but none by viral culture ($P<0.0005$). When samples were taken from patients at predetermined moments without clinical symptoms of RTI, a respiratory virus could be detected in 9% of recipients of SCT by real-time PCR opposed to 1% by viral culture ($P<0.0001$). This outcome suggests that shedding of respiratory viruses in immunocompromised patients can occur. We conclude that respiratory viruses are a major cause of upper and lower respiratory tract disease in recipients of SCT and that real time PCR is superior to viral culture in the detection and follow-up of respiratory virus infections.

INTRODUCTION

Pneumonia is one of the most common infectious complications of stem cell transplantation (SCT). During the past decade, respiratory virus infections have increasingly been recognized as important causes of severe pneumonia in patients who have undergone SCT [1-3]. In these patients, respiratory syncytial virus (RSV) and parainfluenza virus (PIV) have been associated with severe lower respiratory infections after SCT, causing severe morbidity and mortality despite antiviral treatment with ribavirin [4-10]. However, it is not clear yet whether respiratory viruses by itself causes lower respiratory tract infection or that respiratory virus infection may predispose patients to additional infections [8,11-13]. A sensitive method to assess the presence of respiratory viruses in the lower airways may be important to delineate the true incidence of pneumonia caused by respiratory viruses. In addition, since several new antiviral agents against respiratory viruses are now available or under development [14-16], a rapid and sensitive method for detecting respiratory viruses is essential to implement immediate antiviral treatment and to prevent or limit nosocomial spread of infection with

respiratory viruses. Also, the incidence of respiratory viruses like rhinovirus and coronavirus may be underestimated with conventional diagnostic methods as viral culture and immunofluorescence.

The real-time reverse-transcription polymerase chain reaction (PCR) has been proven to be an extremely specific, sensitive and rapid method for detection of respiratory viruses and can be implemented in a routine laboratory setting more easily than classical PCR [17,18]. In a previous retrospective study, we demonstrated that nested RT-PCR was far more sensitive than viral culture and antigen testing for the detection of respiratory viruses in adults with hematological cancer and pneumonia [19].

Therefore, to determine the incidence and severity of respiratory virus infections post transplant and to assess the diagnostic value of real-time RT-PCR for the detection of respiratory viruses compared to viral culture, we conducted a prospective study in recipients who underwent autologous or allogeneic SCT.

PATIENTS AND METHODS

Patients. A prospective study was performed at the Department of Hematology of the University Medical Center Utrecht (Utrecht, The Netherlands) from 1 October 1999 through April 2001, after approval by the local ethical committee.

All patients who underwent allogeneic or autologous SCT were asked to participate. Patients who were included were monitored for respiratory viral infections (influenza virus A and B, respiratory syncytial virus (RSV), parainfluenzavirus (PIV) 1-4, rhinoviruses, enteroviruses, human coronavirus OC43 and 229E, and adenoviruses) during 6 months after transplantation. When patients had complaints of respiratory tract infection (rhinorrhea, pharyngitis, laryngitis, cough, sputum production, dyspnea, with or without fever) a nose-throat (NT) swab was taken within 48 hours for virus culture and real-time PCR for detection of respiratory viruses. In addition, NT swabs were obtained, if possible, on day 2-3, day 4-7, day 8-14, and day 15-21 from start of complaints. Samples collected during an episode of RTI are defined as 'diagnostic' samples. In addition, NT swabs were regularly taken from stem cell transplant recipients on admission, and in week 3, 8, 16 and 26 post-transplant to monitor for respiratory viruses in asymptomatic patients and to establish the diagnostic value of detection by real-time PCR. These are called 'surveillance' samples. Diagnostic procedures such as X-rays and computer tomography of the thorax were performed according to the judgement of the treating physician. If indicated, a bronchoscopy with bronchoalveolar lavage (BAL) was performed for detection of bacteria, mycobacteria, fungi, herpesviruses, and respiratory viruses.

Conditioning regimens and transplantation procedure.

Autologous stem cell transplantation. Patient who underwent autologous SCT for acute leukemia received conditioning regimens with either cyclophosphamide followed by 8 Gy of total body irradiation (TBI) or with oral busulphan and cyclophosphamide. Recipients of autologous SCT who were treated for lymphoma or multiple myeloma received the BEAM preparative regimen (BCNU, etoposide, cytarabine, and melphalan) or high-dose melphalan respectively.

Allogeneic stem cell transplantation. Patients were treated with cyclophosphamide 60mg/kg once daily i.v. for 2 days followed by two doses of 6 Gy of total body irradiation (TBI). In case of a voluntary unrelated

allogeneic (VUD) SCT, patients also received anti-thymocyte globulin (ATG) 4 mg/kg i.v. during 5 days before starting with cyclophosphamide. All transplant recipients received partial T-cell depleted (1 to 2 x 10^5 T-cells/kg) donor marrow.

Infection prophylaxis and infection prevention measures. At admission and during hospitalization at least once a week, surveillance samples were taken from the oropharynx, feces and urine. All transplant recipients received antibacterial prophylaxis with oral ciprofloxacin 500 mg twice a day and oral antifungal prophylaxis with amphotericin B tablets 200 mg 4 times a day and fluconazole 50 mg once daily. The anti-microbial regimen was continued until the granulocyte count had increased until more than 0.5 x 10^9/L. For prevention of bacteremia due to α-hemolytic streptococci, patients received intravenously cephalotin 1 gram 6 times a day post-transplant during neutropenia. Recipients of SCT routinely used valaciclovir 500 mg twice daily and cotrimoxazole 480 mg once a day during the first 12 months post-transplant. In addition, patients who had a positive cytomegalovirus pp65 test (IEA-CMV) during the first 3 months after allogeneic SCT received pre-emptive therapy with ganciclovir [20]. Hospitalized patients were nursed in a single room with free entry for staff and visitors. Careful handwashing and the use of low count microbial food were the only preventive measures used in these patients.

Definitions of respiratory tract infection. Respiratory tract infections were considered to be hospital acquired if symptoms developed ≥ 4 days after admission. An upper respiratory tract infection (URTI) was defined by clinical symptoms as rhinorrhea, pharyngitis and laryngitis, and a lower respiratory tract infection (LRTI) or pneumonia was defined by the development of radiographic pulmonary abnormalities in patients with signs and symptoms such as cough, dyspnea, sputum production, and fever.

Diagnostic methods for routine detection of respiratory viral pathogens. BAL samples and nose/throat (NT) swabs were placed in a tube containing virus transport medium, immediately transported to the laboratory, and processed directly or stored at 4°C for a maximum of 24 hours. The NT and BAL samples were vortexed for 10 s and centrifuged at 2,000x *g* for 15 min. Part of the supernatant was used for conventional virus culture and shell vial culture. The remaining material was stored at -70°C until further analysis by real-time RT-PCR. Conventional viral cultures were performed by inoculating HEP-2C, R-HELA, and tertiary monkey kidney (t-MK) cells with 100 µl of each clinical sample for the detection of respiratory viruses (adenoviruses, parainfluenza viruses, RSV, influenza viruses and picornaviruses). The cultures were examined twice weekly for 10 days for cytopathic effect. In positive cultures, virus was identified by immunofluorescence with monoclonal antibodies (Dako Imagen) for adenovirus, influenzaviruses A and B, RSV A and B, and parainfluenza viruses 1-3. Rhinoviruses were distinguished from enteroviruses through acid-liability testing.

In shell-vial cultures an immunofluorescence test was performed after 1-2 days of culture, usually before a cytopathic effect was noticed using the above-mentioned monoclonal antibodies. BAL samples were also processed for routine bacterial, mycobacterial and fungal culture, and for examination of herpesviruses.

Viral RNA extraction, cDNA synthesis and real-time PCR. A real-time RT-PCR on the stored NT swabs and BAL samples was performed for influenzavirus A and B, parainfluenza virus 1-4, rhinoviruses, enteroviruses, respiratory syncytial virus A and B (RSV), human coronaviruses OC43 and 229E and adenoviruses. RNA was extracted from 100 µl of patient material according to the method of Boom et al. using the MagnaPure LC Total Nucleic Acid Kit (Roche Diagnostics, Mannheim, Germany) [21]. The cDNA synthesis was performed as described previously and the cDNA was subsequently stored at -70°C before real-time PCR [18].

All the primers and probes were selected from GenBank and based on genomic regions of high conservation: the matrix gene was used for influenza A virus [18], the haemagglutinin gene for influenza B virus [18], the 5'-noncoding region for the picornaviruses [22], the N-gene for RSV A and B and coronaviruses 229E and OC43, the haemagglutinin-neuraminidase glycoprotein gene for parainfluenza 1-4 and the hexon gene for the adenoviruses. The primer and probe concentrations were optimized and the real-time Taqman PCR was performed as described previously [18,22]. Briefly, samples were assayed in duplicate in a 25 µl reaction mixture containing 5 µl of cDNA, 12.5 ul 2xTaqMan Universal PCR Master Mix (PE Applied Biosystems), 300-900 nM of the forward primers, 300-900 nM of the reverse primers and 100-200 nM of each of the probes. To control for correct isolation and amplification all samples were spiked before extraction with internal control virus (murine encephalomyocarditis virus [RNA virus] and phocine herpes virus [DNA virus]) [23]. The fluorogenic probes recognizing the human respiratory viruses were all labeled with the 5' reporter dye FAM and a 3' quencher dye TAMRA, whereas the fluorogenic probes recognizing the internal control viruses were all labeled with the 5' reporter dye VIC and the 3' quencher dye TAMRA. By using these different fluorogenic labels, amplification of a human respiratory virus can be distinguished from amplification of the internal control virus.

Statistical analysis. Descriptive statistics were expressed a median values. $?^2$ Analysis using McNemar or Fisher's exact test was performed to determine the degree of significance between the various variables.

RESULTS

A total of 82 patients underwent SCT during the study period. Ten patients were excluded due to several reasons: 6 patients refused participation, 3 patients had follow-up after transplantation elsewhere and 1 patient underwent two transplantations during the study period and was only included once. The 72 included patients had a complete follow-up after SCT for 6 months (or less than 6 months in case of early mortality). Patient characteristics are shown in Table 1. Thirty-seven patients (51%) underwent allogeneic SCT with an HLA-identical sibling donor, 17 patients (24%) received a transplant from a matched unrelated donor, and 18 patients (25%) underwent autologous SCT.

A total of 56 episodes of upper or lower respiratory tract illnesses occurred in 43 patients. No nose-throat swabs were obtained during 4 episodes. Therefore, 52 episodes in 40 patients were evaluable for the detection of a respiratory virus.

A comparison between episodes of respiratory tract illnesses with or without respiratory virus is shown in Table 2. Six episodes of respiratory disease occurred during neutropenia (11.5%). In 23 (57.5%) and 15 (37.5%) episodes of respiratory tract illnesses respectively, recipients of allogeneic SCT used immunosuppressive agents and/or experienced graft-versus-host disease. Thirty of 52 (58%) episodes occurred during winter time (October-March). Most episodes were defined as community acquired (40 out of 52, 77%).

Table 1. Patient characteristics

Characteristics	
No of patients	72
Male/female	49/23
Age, median year (range)	44 (18-64)
Underlying disease	
- AML	15
- ALL	14
- CML	16
- NHL	10
- MM	10
- MDS	3
- AL amyloidose	2
- SAA	1
- M. Hodgkin	1
Type of transplantation	
- allogeneic SCT	37 (51%)
- allogeneic VUD SCT	17 (24%)
- autologous SCT	18 (25%)
Evaluable patients with respiratory tract illness	40 (56%)

AML: acute myeloid leukemia, ALL: acute lymphoblastic leukemia, CML: chronic myeloid leukemia, NHL: non-Hodgkin's lymphoma, MM: multiple myeloma, MDS: myelodysplastic syndrome, SAA: severe aplastic anemia, SCT: stem cell transplantation, VUD: voluntary unrelated donor, RTI: respiratory tract infection, URTI: upper respiratory tract infection, LRTI: lower respiratory tract infection

Table 2. Comparison between episodes of respiratory tract illnesses with or without detectable respiratory virus*.

	episodes with respiratory virus (n=33)	episodes without respiratory virus (n=19)	total episodes (n=52)
Transplantation type			
allogeneic related	19 (58%)	12 (63%)	31 (60%)
allogeneic unrelated	7 (21%)	2 (11%)	9 (17%)
autologous	7 (21%)	5 (26%)	12 (23%)
URTI	25 (76%)	16 (84%)	41 (79%)
LRTI (defined as pneumonia)	8 (24%)	3 (16%)	11 (21%)
Neutropenia	6 (18%)	0†	6 (11.5%)
Immunosuppressive therapy	16 (48%)	7 (37%)	23 (57.5%)
Graft-versus-host-disease	10 (30%)	5 (26%)	15 (37.5%)
Nosocomial/community acquired	10/23	2/17	12/40
Seasonality 'winter'/'summer'	21/12	9/10	30/22
Weeks after SCT (median)	8	13	8

* None of the differences between the episodes were significant, †P=0.075
URTI: upper respiratory tract infection, LRTI: lower respiratory tract infection, SCT: stem cell transplantation, 'winter': from October until March, 'summer': from April until September.

Detection of respiratory viruses during episodes of respiratory tract illnesses. A respiratory virus infection was found by virus culture in 11 of 40 (27.5%) transplant recipients with a respiratory illness during the study period and in 29 of 40 patients (73%) by means of real-time PCR ($P<0.0001$).

A total of 153 nose-throat (NT) swabs and 11 BAL samples were taken during 52 episodes of respiratory tract infection. With conventional virus culture a respiratory virus was isolated in 11/52 episodes (21%, Table 3). Interestingly, when real-time PCR was used, an additional 22 respiratory viruses could be detected, resulting in an incidence of respiratory viruses of 63% (33 out of 52, $P<0.0001$). In three episodes more than one respiratory virus was detected. The most frequently detected respiratory viruses were rhinoviruses (19 out of 33 episodes, 58%). Adenoviruses and coronaviruses were only detected by real-time PCR.

Upper respiratory tract infection. Seventy-nine percent of episodes of respiratory tract infection (41 out of 52) were due to an URTI (Table 3). Twenty-five of these could be associated with a respiratory virus by real-time PCR versus only 10 episodes in case of viral culture (P<0.0001).

Table 3. Respiratory viruses detected either by virus culture or by real-time PCR in 52 episodes of respiratory tract disease

RTI: respiratory tract infection; URTI: upper respiratory tract infection; LRTI: lower respiratory tract infection, RSV: respiratory syncytial virus

	episodes of URTI n=41		episodes of LRTI n=11		total episodes of RTI n=52	
	virus culture	PCR	virus culture	PCR	virus culture	PCR
respiratory virus	10 (24%)	25 (61%)*	1 (9%)	8 (73%)†	11 (21%)	33 (63%)‡
double-infection	-	2	-	1	-	3 ¥
rhinovirus	7 (17%)	16 (39%)	-	3 (27%)	7 (13%)	19 (37%)
influenza virus	1 (2%)	2 (5%)	-	-	1 (2%)	2 (4%)
parainfluenza virus	-	2 (5%)	1 (9%)	1 (9%)	1 (2%)	3 (6%)
RSV	1 (2%)	3 (7%)	-	3 (27%)	1 (2%)	6 (12%)
human coronavirus	-	2 (5%)	-	2 (18%)	-	4 (8%)
adenovirus	-	1 (2%)	-	-	-	1 (2%)
enterovirus	1 (2%)	1 (2%)	-	-	1 (2%)	1 (2%)

*$P<0.0001$, †$P=0.008$, ‡$P<0.0001$
¥: 3 patients had a double infection with a respiratory virus: RSV/rhinovirus; rhinovirus/adenovirus; parainfluenzavirus/rhinovirus

Rhinovirus was detected as the predominant respiratory virus in URTI (39%). None of the patients had progression to a lower respiratory tract infection (LRT) after URTI, and all patients recovered completely without the need for antiviral therapy.

Lower respiratory tract infections. Eleven out of 52 episodes (21%) were considered to be LTRI. All patients with a LRTI were admitted to the hospital. Fever, cough, dyspnea and malaise were the predominant signs and symptoms in these patients; none of them had sequelae of an URTI. In 10

episodes bronchoscopy with BAL was performed in addition to NT swabs. By viral culture a virus infection was diagnosed in one episode (parainfluenza virus 4), but by real-time PCR in 8 of 11 episodes (73%, P=0.008, Table 2). In one patient the BAL sample contained both a rhinovirus and a parainfluenza virus. Another patient whose BAL sample contained a rhinovirus had developed respiratory failure primarily after treatment with rituximab for post transplantation lymphoproliferative disease. Irradiation pneumonitis, invasive aspergillosis, and bronchiolitis obliterans were diagnosed by biopsy in the three remaining patients with pulmonary abnormalities whose BAL fluid or NT swabs remained negative for a respiratory virus. None of the recipients of SCT with a LRTI was diagnosed to have a bacterial infection according to the microbiological results of the BAL samples. Four patients with pulmonary abnormalities died: one patient due to invasive aspergillosis, one patient who had been treated with rituximab for post-transplantation lymphoproliferative disease, but who also had a positive PCR for rhinovirus, and two patients in whom only a coronavirus and RSV were detected. The death of the last two patients was considered as respiratory virus associated mortality (2 out of 8 episodes [25%] of LRTI in which respiratory viruses were detected). Both patients had been treated with aerosolized ribavirin.

Table 4. Differences between allogeneic sibling SCT, voluntary unrelated SCT, and autologous SCT *

	allogeneic SCT n=37	VUD SCT n=17	autologous SCT n=18
Patients with RTI	23 (62%)	7 (41%)	10 (56%)
Episodes of RTI	31	9	12
respiratory virus (virus culture)	5 (16%)	4 (44%)	2 (17%)
respiratory virus (RT-PCR)	15 (48%)	7 (78%)	7 (58%)
Neutropenia (<0.5 . 10^9/L)/ episode	2 (6%)	3 (33%)	1 (8%)
Immunosuppressiva/ episode	20 (65%)	3 (33%)	NA
GVHD/ episode	13 (42%)	2 (22%)	NA

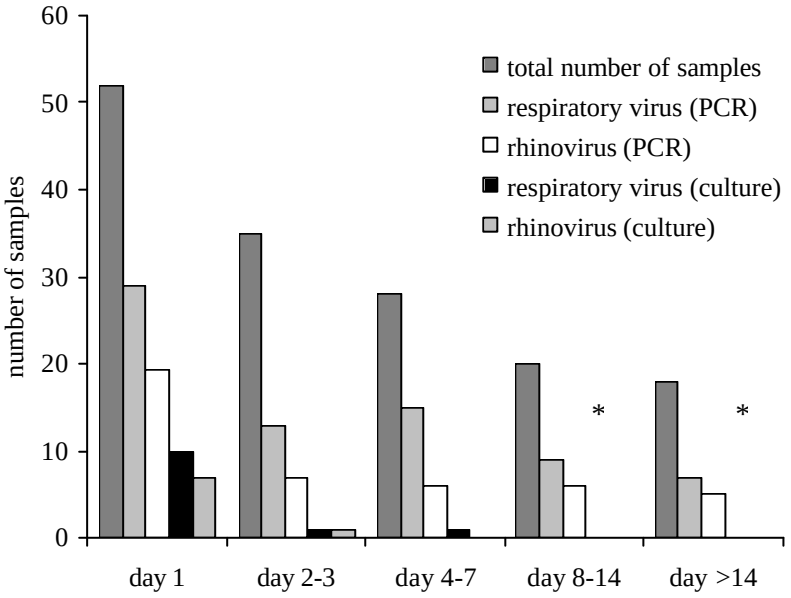
SCT: stem cell transplantation; VUD: voluntary unrelated donor; RTI: respiratory tract infection; GVHD: graft-versus-host-disease; NA: not applicable
* None of the differences between the types of transplant were significant.

Differences between allogeneic sibling SCT, matched unrelated SCT and autologous SCT. There was no significant difference in incidence of respiratory tract infections between the three different SCT modalities (Table 4). More patients who underwent unrelated SCT had severe neutropenia during an episode of respiratory tract infection, but the number of patients is too small to draw conclusions.

Also, there were no significant differences between allogeneic sibling and unrelated SCT in percentages of patients who received immunosuppressive agents or who had developed GVHD.

Duration of viral detection in episodes of respiratory tract illnesses. Follow-up NT samples have been taken in 36 of 52 episodes of respiratory tract infection (69%). As is shown in figure 1, respiratory viruses were detected by real-time PCR over a longer period of time than by virus culture. In samples obtained one week after start of complaints from patients who had a respiratory virus disease, the initial respiratory virus could still be detected by means of real-time RT-PCR in 16 out of 25 (64%) samples, but none by viral culture (P=<0.0005). Rhinovirus was detected as the predominant virus (11 of 16 samples, 69%), next to coronaviruses (n=2), RSV (n=2) and enterovirus (n=1). In addition, as was detected in follow-up samples of 1 patient with a rhinovirus-associated URTI, a new respiratory virus had emerged (adenovirus). The patient had no additional complaints.

Figure 1. Follow-up samples of patients with a respiratory illness, comparing virus culture with real-time PCR for outcome of a respiratory virus.



* Follow-up cultures > 7 days of respiratory virus infection: by real-time PCR: 16/25 of the samples positive and by viral culture: 0/25 (*P*<0.0005)

Detection of respiratory viruses in the absence of respiratory tract illness. A total of 259 surveillance NT swabs were taken from the 72 patients. By real-time PCR a respiratory virus could be detected in 24 samples (9%) of recipients of SCT without signs and symptoms of a viral respiratory tract illness. By virus culture only 3 samples were positive (1%, P<0.0001, Table 5). Rhinovirus was the predominant pathogen in these surveillance samples (21 out of 24 samples, 87.5%), but coronavirus (2 times) and adenovirus (3 times) were also detected. Surveillance NT swabs taken before SCT were positive for a respiratory virus in 9 patients (rhinovirus: 8, coronavirus: 1); they all were without complaints at the time of collection. Three of these patients developed an URTI with the initially detected virus (rhinovirus) within one month. Prolonged detection (2 respectively 3 months) of rhinovirus during surveillance without causing respiratory disease was observed in 2 of these patients.

Table 5. Outcome of surveillance samples (n=259)

	positive surveillance NT samples with virus culture (n=3)	positive surveillance NT samples with real-time PCR (n=24)
rhinovirus	2	21*
RSV	-	-
human coronavirus	-	2
adenovirus	1	3
influenzavirus	-	-
parainfluenzavirus	-	-
enterovirus	-	-

NT: nose-throat, RSV: respiratory syncytial virus. * *P*<0.0001, 2 samples contained 2 respiratory viruses: adenovirus/rhinovirus and coronavirus/rhinovirus.

DISCUSSION

Our prospective study showed that respiratory viruses are a major cause of respiratory tract disease in adult recipients of SCT. We found an incidence rate of upper or lower RTI associated with a viral infection of 21% by conventional viral culture and 63% by real-time PCR in 72 SCT recipients during a period of six months from SCT. In a previous retrospective study we also observed a significant increase in incidence of respiratory virus infections in immunocompromized patients when a nested RT-PCR was compared with viral culture [19]. Reported incidences of respiratory virus infections in adult stem cell transplant recipients with acute upper and lower respiratory illnesses vary in literature from 3.5% to 36%. In these studies viral culture or direct immunofluorescence on nose-throat swabs, nasopharyngeal aspirates or BAL specimens were used [1-3].

Our results showed that respiratory viruses were mainly associated with upper respiratory tract infections, of which rhinovirus was the predominantly detected respiratory virus. This outcome is not surprisingly, since also immunocompetent adults have 2-4 common colds per year of which

rhinoviruses account for about 30–50% of all respiratory illnesses [24]. In contrast with other studies [1-3,25], we did not observe that SCT recipients with an URTI had progression to a LRTI. All patients who were diagnosed with a LRTI were having clinical features of pneumonia without obvious complaints of a viral URTI, indicating that in immunocompromised patients respiratory viruses may cause pneumonia not preceded by an URTI.

We have shown that real-time PCR is superior to virus culture for the detection of respiratory viruses in lower respiratory tract. We found 9 respiratory viruses in 8 BAL specimens by real-time PCR compared to only one by virus culture. The negative results in the detection of respiratory viruses in BAL specimens by viral culture may be explained by the time, which had elapsed between the initial infection with a respiratory virus and the occurrence of a LRTI clinically. We have not diagnosed any bacterial infections in recipients of SCT with a LRTI, which may be explained by the use of antimicrobial prophylaxis or by the administration of broad-spectrum antibiotics prior to bronchoscopy with BAL. On the other hand, the high incidence of respiratory pathogens that we have detected in LRTI may indicate that respiratory viruses may be more important as causative pathogens of LRTI than bacteria. We observed no difference between the incidence of respiratory viral illness and the type of transplantation, although recipients of voluntary unrelated donor SCT tended to be more at risk for developing respiratory virus infection during neutropenia as is reported before [8].

Rhinoviruses were identified both by viral culture and by real-time PCR as the major cause of respiratory virus infection, and predominantly in upper respiratory tract infection. However, in three patients with a LRTI a rhinovirus infection was detected (once in combination with a parainfluenza virus infection). Rhinoviruses have been described before in several studies as causative pathogens of LRTI in immunocompromised patients [12,19,26], either as the sole pathogen or as a co-pathogen next to bacteria or other respiratory viruses. Yet, no or only a very low number of rhinovirus infections in both URTI and LRTI was observed in 2 recent studies that prospectively investigated the incidence of respiratory virus infections in hematological cancer patients [2,25]. The results of these two studies are rather surprising, since rhinoviruses are the most common viruses isolated from immunocompetent persons with acute upper respiratory illnesses and are well known to cause occasionally LRTI in older adults and neonates [24,27]. The role of rhinovirus as the causative pathogen of pneumonia is not clear yet. The virus may have a direct cytopathogenic effect or act indirectly through immunologic responses, which may predispose infection by other pathogens. In several studies rhinoviruses or replicative-strand rhinoviral RNA have now been recovered from the lower airways, suggesting that rhinoviruses may have a direct cytopathogenic effect [11,13]. Several studies including ours have now indicated that rhinovirus infection may be a serious threat in immunocompromised patients. Thus, rapid detection and subsequent treatment of rhinovirus infection may be important, even more so now that recent studies suggest that the antiviral drug pleconaril could be effective in the treatment of severe picornavirus infections including rhinoviruses in SCT recipients [16].

We detected 6 times RSV more frequently by real-time PCR than by viral culture, but it is also well known that RSV viral loads in adults are usually low. Fifty percent of the patients with RSV infection presented with a LRTI, which is in concordance with results of previous studies [2,10,28]. One patient with RSV pneumonia died, despite treatment with aerosolized ribavirin.

Of interest is the number of human coronaviruses we detected by real-time PCR. Human coronaviruses, types 229E and OC43, are a major cause of the common cold, but they are notoriously difficult to culture. These viruses, however, may cause incidentally pneumonia in both immunocompetent and immunocompromised patients [19,27,29]. We found coronavirus as the probable cause of illness in 8% of episodes of respiratory tract disease. Half of the patients with a coronavirus infection developed a LRTI and one patient died as a result of a coronavirus pneumonia. In contrast with our results, a recent study did not detect any human coronavirus RNA in BAL samples from patients who had undergone SCT [12]. Presently, a new human coronavirus has been discovered as the causative pathogen of an outbreak of severe acute respiratory syndrome, indicating that these viruses may also infect the lower respiratory tract [30].

Respiratory virus infection due to parainfluenza virus occurred in 3 patients and was clustered in the same week in 2000; all were community-acquired. Besides this small epidemic of parainfluenza virus, no other epidemics occurred during the study period. Only 2 patients had an URTI due to influenza virus.

Although we detected adenovirus in 3 surveillance samples and in follow-up samples of one patient with a rhinovirus infection, no respiratory tract infections due to adenovirus were detected in our study population. Adenoviruses have been recognized as a serious pathogen in SCT recipients, especially among pediatric patients [31]. But, adenovirus infections can also be a threat to adults undergoing SCT as has been shown in two recent prospective studies [32,33]. T-cell depletion, GVHD grade 4 and unrelated or mismatched transplants are associated with a higher risk of developing adenoviral disease [31-33]. We have no clear explanation for the low incidence of adenovirus infection in our study. All patients with an allogeneic transplantation received T-cell depleted stem cells, however the incidence of severe GVHD in our population was low (only one patient developed GVHD grade 3-4). Another reason could be that we only took samples from the respiratory tract to detect adenovirus. A lack of sensitivity of our real-time PCR is unlikely as primers and probes were selected to react with all serotypes, and the detection threshold is approximately 500 copies/ml.

To establish the diagnostic value of real-time PCR, we also analyzed NT swabs of SCT patients collected at moments when patients did not have complaints of a respiratory tract infection ('surveillance' samples). By real-time PCR we found respiratory viruses in 9% of these samples, of which 88% were rhinoviruses. In contrast, a virus infection was found in 63% of samples from patients with symptoms of RTI, of which 58% were rhinoviruses. This indicates that the detection of respiratory viruses by real-time PCR in samples from SCT patients with RTI has diagnostic significance, also for rhinoviruses. Most of the surveillance samples in which rhinoviruses were

detected, were taken before transplantation at admission to the hospital. In accordance with our results, immunocompromised patients are known to shed virus for a longer period of time [34]. Therefore, these positive surveillance samples could reflect the long-term shedding of rhinovirus after earlier infection. Surprisingly, we did not find a usual seasonal pattern in surveillance samples positive for rhinovirus among our SCT recipients. It would be interesting to determinate the geno- or serotypes of the rhinoviruses to know more about the circulation of these respiratory viruses in immunocompromised patients. A recent study showed striking genetic diversity of rhinovirus strains circulating in the community, some of which were strictly seasonal, whereas other variants were detected during several seasons [35].

In summary, the present study has shown that respiratory viruses are a major cause of respiratory tract disease in stem cell transplant recipients. We have demonstrated that real-time PCR is much more sensitive than viral culture and antigen testing for detection of respiratory virus infection in recipients of SCT, especially in case of lower respiratory tract infection. This outcome is of importance, since the use of a sensitive and rapid method such a real-time PCR would permit fast and early diagnosis of respiratory virus infections in immunocompromised patients, and thus would allow early initiation of antiviral treatment and of preventive measures. Rhinovirus caused the majority of URTI, but one must be aware that rhinoviruses can also be detected occasionally in asymptomatic patients, suggesting that persistent shedding of respiratory viruses can occur in immunocompromised patients

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Chapter 9

Enhanced severity of viral respiratory tract infection in asthma patients is not associated with delayed viral clearance and viral load

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INTRODUCTION

Viral respiratory infections, particularly rhinovirus infections, are the most common cause of asthma exacerbations. Johnston et al. reported that viral infection was associated with 80%-85% of asthma exacerbations in 9 to 11 year old children [9]. Picornaviruses (human rhinoviruses (HRV) and enteroviruses) accounted for about 65% of these viral respiratory asthma exacerbations. Also in adult asthma patients respiratory virus infections are associated with the majority of exacerbations [12].

In addition, Corne et al saw that HRV infection usually leads to more severe and longer duration of lower respiratory tract symptoms in asthma patients than in otherwise healthy individuals [2]. It has been suggested that the rhinovirus-specific intracellular adhesion molecule 1 (ICAM-1) receptor is upregulated in asthma patients, and that asthma patients are therefore more susceptible to HRV infection [11,11,20] . Another more general mechanism might be the delayed clearance of the virus in asthmatic individuals as compared to otherwise healthy persons, due to a difference in the immunological response. In experimental inoculation studies with rhinovirus it was shown that within a group of patients with allergic rhinitis or asthma, those who showed a high IFN-y –IL-5 ratio (Th1 helper response) tended to have less severe symptoms, and shed less virus compared to those who had a low IFN-?–IL-5 ratio (Th2 helper response) [5]. This could imply that anti-viral therapy could reduce the prolonged shedding of the virus in asthma patients and thereby reduce symptoms. However, the exact mechanisms of viral contribution to exacerbations in naturally occurring virus infection must still be elucidated.

The purpose of the here described study was to assess magnitude and duration of viral replication and severity of symptoms in relation to various cytokines in nasal washes in asthmatics and non asthmatics during different naturally occurring respiratory virus infections. Therefore, we longitudinally followed asthmatic individuals and healthy controls during viral respiratory tract infection.

PATIENTS AND METHODS

Subjects. This was a prospective study performed from September 1998 through August 2000, after approval by the local ethical committee. We recruited 44 people with asthma, aged 18-45 years, from family doctor's practices. Asthmatic patients, diagnosed as such according the Dutch asthma guidelines, showing a reversibility in peak expiratory flow (PEF) of ≥ 15% and/or forced expiratory flow in 1 second (FEV1) of > 9% [3]. All patients were treated with various combinations of inhalation therapy, such as inhaled corticosteroids, ß-agonists and ipratropium bromide. We also enrolled 44 healthy control subjects without history of asthma and/or allergy. Written informed consent was obtained from all participants.

Procedures. Both asthmatic and control subjects daily recorded signs and symptoms of the upper respiratory tract (URT) and of the lower respiratory tract (LRT), rated from 1 (mild) to 3 (severe). URT symptoms were defined as running nose, sneezing, stuffy nose, itchy nose, watery/sore eyes, sore throat, hoarse voice and headache. LRT symptoms included coughing, wheezing, difficult breathing and shortness of breath during effort. Asthmatic participants recorded peak expiratory flow (PEF) twice daily. All participants were asked to contact the investigators if the URT symptoms totaled 4 or more, and/or if the LRT symptoms exceeded

5. The subjects were then visited within 48 hours. Follow-up visits were performed at day 3/4, day 5/6, after 2 weeks and after 8 weeks (baseline). The duration of URT and LRT symptoms was determined by the number of days with an URT or LRT symptom score of 1 above the individual baseline score respectively. The severity of the URT and LRT symptoms was defined as the maximum of URT and LRT symptom scores respectively. During each visit a nasal wash (NW) was collected from each nostril as described previously [7]. Briefly, 10 ml of sterile saline was instilled in each nostril and collected in the same tube. The tube was mixed thoroughly and placed on wet ice immediately after. One ml of NW was used for virus culture. The remnant was mixed with an equal volume of diluted sputolysin reagent (6.5 mM dithiothreitol in 100 mM phosphate buffer) (Calbiochem-Novabiochem Corporation, San Diego, CA) to solve mucus and facilitate the analysis. Both aliquots were then clarified by centrifugation (1000 *g* for 10 min at room temperature). The remaining supernatant fluid was stored at -80°C until further analysis.

Respiratory virus detection and quantification. Conventional viral cultures were performed by inoculating HEP-2C, R-HELA, and tertiary monkey kidney (t-MK) cells with 1 ml NW for the detection of respiratory viruses (parainfluenza viruses 1-3, respiratory syncytial virus (RSV) A and B, influenza viruses and picornaviruses). The cultures were examined twice weekly for 10 days for cytopathic effect. In positive cultures, virus was identified by immunofluorescence with monoclonal antibodies (Dako Imagen) for influenza viruses A and B, RSV A and B, and parainfluenza viruses 1-3. HRV were distinguished from enteroviruses through acid-liability testing. In shell vial cultures an immunofluorescence test was performed after 1-2 days of culture, usually before a cytopathic effect was noticed, using the above mentioned monoclonal antibodies.

A real-time quantitative RT-PCR was performed on the stored supernatant fluids for influenza virus A and B, parainfluenza virus 1-4, picornaviruses, RSV A and B and human coronaviruses OC43 and 229E as described previously [19]. The number of viral RNA copies for influenza A and B, RSV A and B and rhinovirus in the clinical samples was determined by extrapolation to a standard curve generated upon amplification of serial dilutions of electron microscopically (EM)-counted virus stocks. To estimate the quantity of human coronaviruses OC43 and 229E, for whom no EM-counted virus stocks were available, viral particles were expressed as relative units (RU). Every amplification cycle represents a 2-fold increase in the number of viral RNA copies. The viral load was expressed as 2-fold increase per cycle relative to a baseline value of 2 copies at threshold cycle 36 ($RU= 2^{36\text{-threshold cycle}}$).

Assays for cytokines. IFN-?, IL-6, IL-8 (CLB, Amsterdam, The Netherlands) and IL-5 (BioSource International, CA, USA) were measured using commercially available human cytokine ELISA kits according to the manufacturer's recommended protocol. The supernatants were assayed in duplicate and the results were expressed in pg/ml. The limits of sensitivity of these assays, as supplied by the manufacturers, were as follows: IFN-? 1 pg/ml, IL-5 4pg/ml, IL-6 0.2 pg/ml, and IL-8 1 pg/ml.

Statistical analysis. Analyses were based on data collected from infected subjects. Only one period of infection per subject was used for analysis. Differences between asthmatic and control groups were analyzed by Mann-Whitney U test (SPSS 11.0 for windows). Comparison of measures of infection and illness (viral load, symptom assessment and peak flow measurements) and cytokine levels between asthmatics and controls were done using the Wilcoxon signed rank test (SPSS 11.0 for windows). Correlations were determined using the Spearman's rank correlation coefficient (SPSS 11.0 for windows).

RESULTS

Study population and episodes of viral RTI. A total of 44 asthmatics and 44 control subjects were enrolled in the study. Out of these 88 persons, 43 persons reported a total of 57 episodes of respiratory tract illness. A respiratory virus was detected by PCR in 33 out of 57 (58%) episodes. Viral culture gave no added diagnostic value. For each patient only one episode of respiratory virus infection was used for subsequent analyses, resulting in 14 episodes of both asthmatic patients and non-asthmatic individuals. Patient characteristics are shown in table 1.

Table 1. Patient characteristics

	Asthmatic group	**Control group**
No. of patients	14	14
Sex (male:female)	4:10	5:9
Age (median;range)	38.5 (26-44)	33.5 (21-44)
Smoking	2	4
Influenza vaccination	9	2

No significant differences were seen between the number of respiratory tract illnesses caused by a respiratory virus in asthmatic (16/33, 48%) and control (17/33, 52%) participants (table 2.). The majority of infections (n=33) were caused by HRV. Nine out of 14 asthmatic patients received influenza vaccination. Surprisingly, influenza infection was observed in 5 asthmatic patients as compared to just one in the control group of non-asthmatic individuals. The other viruses observed in both groups were RSV and human coronaviruses.

Symptoms of viral RTI. The duration and severity of URT symptoms were similar for both the asthmatic and the control group. However, the observed respiratory tract infections resulted in significantly more severe LRT symptoms in the asthma patients as compared to the non-asthmatics ($p=0.02$: table 2). The duration of symptoms of the LRT was significantly longer in asthmatic patients as well ($p=0.03$: table 2). Subgroup analysis was performed in the asthmatic population to seek for differences between HRV infection and infection caused by other respiratory viruses. No significant differences were found between these viruses with regard to severity and duration of infection, or fall in PEF.

Table 2. Detected viruses, median viral shedding, duration, and severity of upper respiratory tract (URT) symptoms and lower respiratory tract (LRT) symptoms

	Asthmatic group n=14 (episodes used for analyses)	**Control group** n=14 (episodes used for analyses)
Detected respiratory viruses	16 (14)	17 (14)
- *rhinoviruses*	8 (6)	12 (10)
- *influenza virus A/B*	5 (5)	1 (1)
- *RSV A/B*	1 (1)	1 (1)
- *Coronaviruses*	3 (2)	2 (2)
- *Parainfluenza viruses*	0	0
- *Enteroviruses*	0	0
Days of viral shedding (range)	6 (1-16)	4 (1-9)
Viral load (copies/ml)	20732 (2-62270)	999 (8-262136)
Duration (days) of URT symptoms (range)	9 (5-19)	8 (1-17)
Severity of URT symptoms (range)	8 (4-9)	7 (2-9)
Duration (days) of LRT symptoms (range)	7 (3-17)	3.5 (0-8)*
Severity of LRT symptoms (range)	5 (0-9)	0.5 (0-5) †

* p=0.03
† p=0.02

Viral dynamics. The observed differences in the duration and severity of the symptoms in the asthma patients as compared to the non-asthmatic patients could be associated with a difference in the level or duration of virus production. By using a quantitative real-time PCR for the detection of the different respiratory viruses we determined the dynamics of the different viral infections. Interestingly, no significant differences were observed for either the maximum levels of virus produced or the duration of detection of viral RNA as compared between asthma patients and non-asthmatic individuals (table 2). In addition, the peak virus load and the duration of viral detection were not correlated to duration and severity of symptoms of the LRT either. These data indicate that the observed differences in LRT symptoms between asthma patients and non-asthmatic individuals can not directly be explained by either the level or duration of virus production. Interestingly, if the dynamics of the virus infections in asthma patients is compared to the peak flow reduction and the LRT symptoms, it seems clear that the actual viral infection precedes the peak flow reduction and LRT symptoms (fig. 1-2).

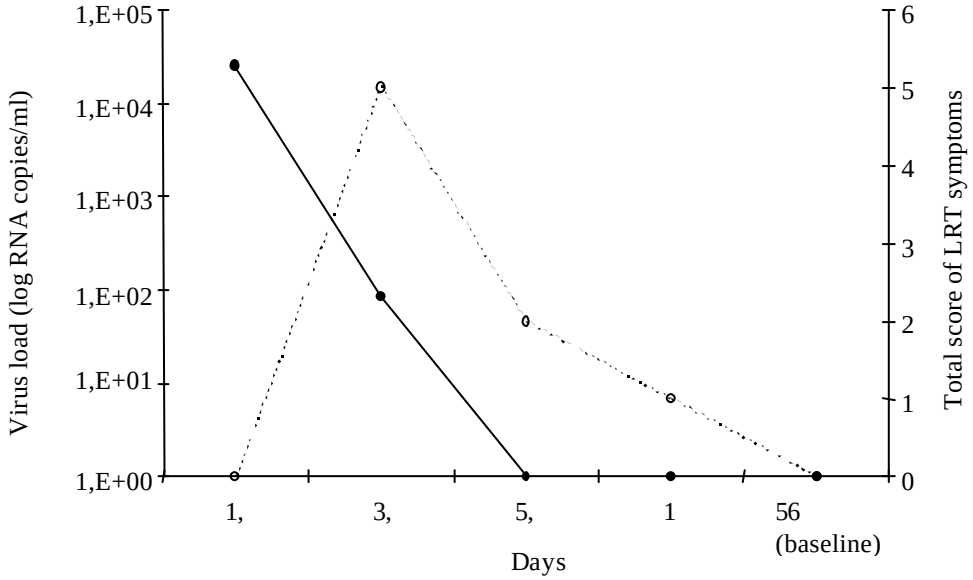

Figure 1. Median virus load (•) and lower respiratory tract (LRT) symptoms (o) in asthmatic subjects (n=14)

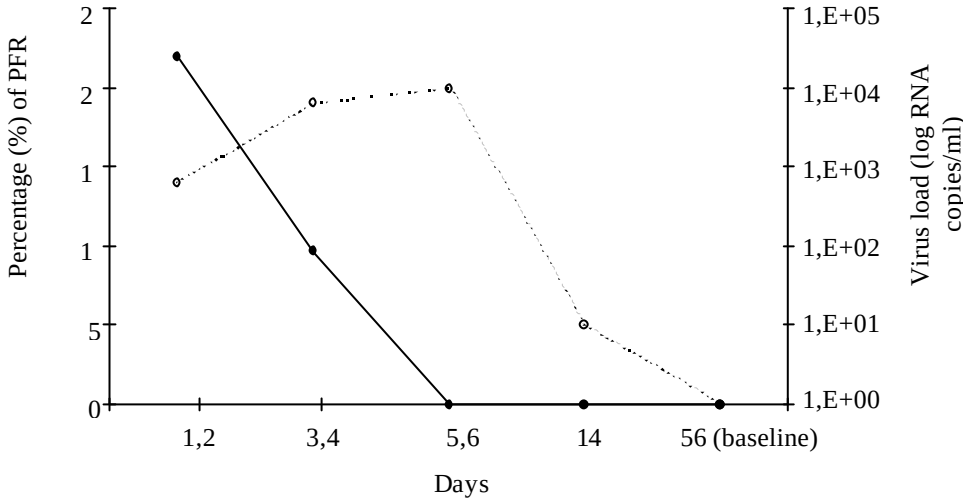

Figure 2. Median virus load(•) and peak flow reduction (PFR) (o) in asthmatic subjects (n=14). Reduction of peak flow is defined as percentage reduction from personal median baseline score.

Inflammatory cytokine response. Investigation of the inflammatory response demonstrated that in both groups the levels of the pro-inflammatory cytokines IL-6 and IL-8 were significantly increased at day 3-4, as compared to baseline (table 3). These increases in IL-6 and IL-8 were observed at the moment that the virus load already returned to baseline, while asthma patients suffered from LRT symptoms and peak flow reductions (fig. 3). The levels of IL-6 perfectly paralleled the LRT symptoms

and peak flow reduction. No significant differences of IL-6 or IL-8 could be determined between both study groups during signs and symptoms of RTI. Neither the levels of IL-5 nor IFN-? present in the

Table 3. Median cytokine levels (pg/ml) in nasal washes of asthmatic and control patients at different time-points (range)

	Day 1,2		**Day 3,4**		**Day 5,6**		**Baseline**	
	Asthmatic	control	Asthmatic	control	Asthmatic	control	Asthmatic	control
IL-6	12 (0-322)	0 (0-854)	107 (0-975)*	124 (0-502) ‡	70 (0-613)	42 (0-353)	0 (0-40)	0 (0-16)
IL-8	445 (0-2398)	526 (0-31536)	348 (130-2781)*	2921 (41-40107) ‡	598 (272-3387)	477 (320-52040)	272 (0-1611)	254 (6-4944)

* $p < 0.05$ in comparison to baseline
‡ $p < 0.01$ in comparison to baseline

nasal washes of asthmatics and controls did exceed the detection limit of the assay (4pg/ml and 1pg/ml respectively). Therefore differences in IFN-y-IL-5 ratio could not be assessed.

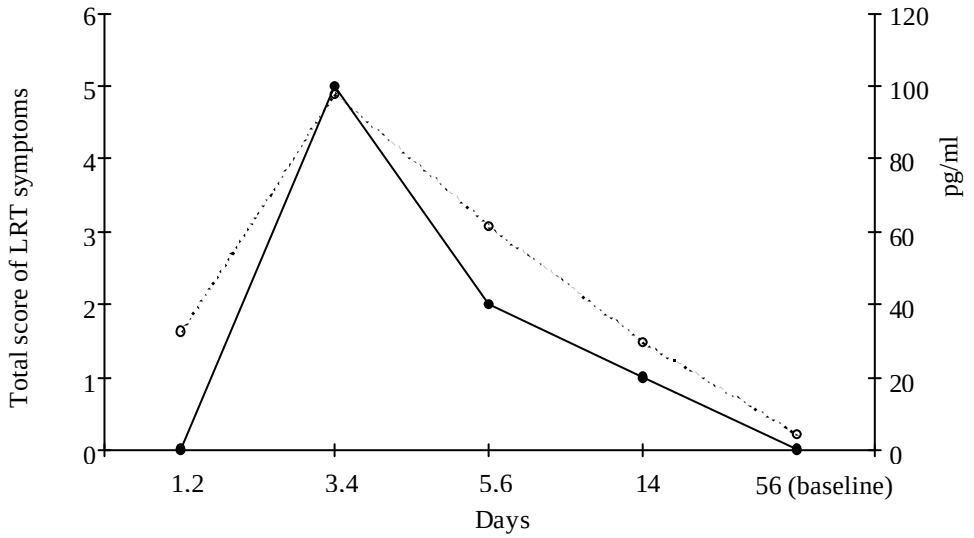

Figure 3. Median lower respiratory tract (LRT) symptom score (•) and IL-6 concentrations (o) in asthmatic subjects (n=14)

DISCUSSION

In this study we found that (i) after the first symptoms of U/LRTI there is a quick clearance of respiratory viruses in the URT in asthma patients as well as healthy controls. In addition, no differences in proinflammatory cytokines could be determined in the nasal washes of asthmatic and non-asthmatic individuals. Secondly (ii) the asthmatic symptoms of the lower respiratory tract persisted for more than 1 week after the clearance of virus.

The percentage of viruses detected from an asthmatic individual during symptoms was approximately 60%. This is in accordance with the amount of viruses detected in previous studies using molecular diagnostics for rhinovirus detection [12,19].

This study demonstrates that viral respiratory infections caused more severe problems of the LRT in asthma patients as compared to healthy controls which confirms the findings of a recent study in patients that were infected with HRV [2]. The fact that the LRT symptoms in the asthmatic group were present for almost a week after the clearance of the virus indicates that there is an ongoing inflammatory response. It has been suggested that the delayed clearance of a virus, caused by a different immunological response, is associated with asthma-exacerbations in asthmatic individuals [5]. As mentioned, we did not see such a prolonged viral infection in the URT of asthmatic patients

Recent studies have mainly focused on the role of HRV infection in asthmatic patients, since HRV is the most frequently detected pathogen in virus-associated asthma exacerbation [4,9,12]. An explanation for the enhanced severity of LRT symptoms in asthmatic patients infected with HRV has

been the Th2 dominancy in asthma patients leading to a more explicit Th2-helper response in asthmatic individuals [5,10,15]. From experimental HRV infection evidence is growing that a typical pattern of cytokine production in an asthma patient may confer a risk of an exacerbation related to a HRV infection [5,16].We could neither detect IFN-y and IL-5 in the NW of our study population. Therefore, no conclusion could be drawn whether the more enhanced severity of LRT symptoms were related to a more explicit Th2-helper response in asthmatic individuals compared to healthy control subjects. Although IFN-y and IL-5 have been detected and analyzed in asthmatic subjects, it has been reported previously that IFN-y and IL-5 levels in induced sputum remain below the detection limits during natural acute respiratory virus infections [17]. IL-8, a potent chemoattractant for and activator of neutrophils, has also been implicated in the pathogenesis of respiratory virus infection and asthma by triggering the inflammation that leads to exacerbations [6,8,13,18]. Although we did see a statistically significant increase in IL-8 as compared to baseline we could not observe a difference in intranasal IL-8 levels between the asthmatic group and the control group.

Finally it has been suggested that an asthmatic exacerbation is associated with a delay in viral clearance, for example due to the Th2 dominant cytokine response, or due to an increased ICAM-1 expression in asthmatic subjects [5]. Our study clearly shows that there was no delay in viral clearance in the upper respiratory tract in the asthmatic individuals as compared to the control subjects. Neither was there a difference in duration and severity of LRT symptoms in the HRV infected asthmatic subjects as compared to the asthmatics infected with other respiratory viruses. This observation, that all different respiratory viruses are able to cause severe LRT symptoms, indicates that there might be a more general, not HRV specific, mechanism of induction of asthma exacerbation, which is not associated with viral load. These results suggest that virus-induced asthma exacerbations are not confined to HRV and that the association with HRV is more related to the fact that HRV accounts for the majority of common-cold illnesses [1].

Interpretation of the results from this study is hampered by the small study population, the large variety of different viruses, and the lack of samples from the lower airways. Studies to determine the mechanisms by which viruses cause lower airway disease are often hampered by difficulty in sampling lower airways. It has been shown that HRV have the capacity of infecting the lower airways [14]. The virus which is present in the upper respiratory tract may also invade the lower airway epithelium in a later stage of the infection thereby causing inflammation and provoke bronchospasm, airway obstruction and wheezing. It is also possible that the virus is largely confined to the upper airway and that remote, indirect mechanisms provoke asthma. How naturally occurring respiratory viral infections provoke asthma remains unknown.

In conclusion, with the use of quantitative real-time PCR we showed that the persistence of the virus, as well as viral load, is not associated with the induction and/or persistence of asthmatic symptoms. Therefore, it is questionable whether anti-viral therapy will be effective after the onset of an exacerbation. Elucidation of the local and systemic inflammatory response in virus-induced asthma

exacerbations remains a challenge, especially for the development of more sophisticated anti-inflammatory strategies.

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Chapter 10

Summary and Discussion

Respiratory viral infection in adults causes significant morbidity and mortality, especially in high-risk patients. They are responsible for a large percentage of physician office visits, days lost from work,utilization of emergency center facilities, and hospitalization.

Clinical diagnosis cannot discriminate between various etiologies. Furthermore, etiologic diagnosis is necessary for effective antimicrobial therapy. The present virological methods such as culture, serology and antigen detection are often insufficient. There is a need for novel methods allowing early rapid diagnosis. In this thesis we describe the development and use of molecular methods (PCR) to increase the ability to diagnose respiratory tract infection (RTI) caused by respiratory viruses and the use of these methods to study the role of these respiratory viruses in different patient populations.

SUMMARY

Diagnosis and antiviral treatment of respiratory virus infection

Traditionally, treatment and prevention of respiratory viral disease has mainly focused on influenza virus infection. The epidemiology, impact and severity of influenza virus infection have been studied extensively and it has been shown that influenza virus infection is associated with significant mortality and morbidity every year. Despite these studies influenza, like other respiratory virus infections is often unjustly considered a relatively harmless infectious disease and treatment as not being necessary. Existing antiviral drugs for the influenza viruses, such as amantadine and rimantadine or the recently developed neuraminidase inhibitors, consequently are seldom prescribed. The limited use of amantadine and rimantadine is probably also due to their side effects and to the rapid emergence of resistant strains. A new class of antiviral drugs with a different mechanism of action, the neuraminidase inhibitors, has recently been developed to prevent (prophylactic use) or to treat (therapeutic use) influenza A and B infections **(Chapter 2)**. Timely treatment with a neuraminidase inhibitor reduces the duration of illness by an approximately 1.5 days in an otherwise healthy population. In addition to a reduced disease burden and a limited transmission, this can be economically advantageous in an otherwise healthy population. The therapeutic use of these antiviral agents is, nevertheless, more complicated. They must be administered in an early phase of the infection to be effective. Flu-like illnesses however are not only caused by influenza viruses but by other respiratory viruses as well. An additional difficulty is that the symptoms of all these respiratory viruses overlap: common cold like illnesses may occur with all these viruses as well as with bacteria. However, at present there is no quick, sensitive, and reliable test available to establish the diagnosis to first-line healthcare professionals, who are mostly confronted with the otherwise healthy population suffering from influenza virus infection. There clearly appears to be a problem in clinical diagnosis as well as in laboratory diagnosis. In **Chapter 3**, we therefore investigated whether it would be possible to establish a diagnosis of influenza on the basis of clinical signs and symptoms only. In the study

population neither the criteria of the International Classification of Health Problems in Primary Care (ICHPPC) nor the sentinel criteria did distinguish satisfactorily between infection by influenza virus or by other viruses/pathogens. The results of our study demonstrate a positive predictive value of 75% and a negative predictive value of 80% for the combination of cough, headache at onset, feverishness at onset, and vaccination status but only during the period with increased influenza activity. We conclude that besides intensification of the surveillance networks and notification of the results to the general practitioner (GP) these clinical signs could be used as a guideline for GP's. Nonetheless, rapid laboratory diagnostic investigation clearly is a prerequisite for effective, efficient antiviral treatment. While clinical diagnosis lacks specificity, conventional laboratory diagnosis however is still laborious and time-consuming.

Real-time quantitative PCR

In the studies described in **Chapters 4-6** we demonstrate that the real-time PCR assays that we have developed allow the sensitive and specific detection of infection by influenza virus A and B, RSV A and B and human coronaviruses (HCoV) 229E and OC43. The accurate calculation of clinical sensitivity for a PCR assay, however, is hindered by the lack of a suitable diagnostic " gold standard". Nevertheless, our studies show that real-time PCR exhibits a significant advantage over the existing conventional diagnostic methods such as (rapid) virus culture in terms of sensitivity, specificity and speed. The real-time PCR assays allow accurate diagnosis within 4-5 hours. The risk of false-positive and false-negative results is minimized by the use of internal controls and positive controls. The described methods in this chapter also have the advantage of a standardized protocol that can easily be applied for other respiratory viruses. The assay can be performed under uniform amplification conditions, thereby using target specific primer and probe sets. In addition, the procedure is less complicated than other RT-PCR methods and the chances of contamination are minimized because there is no post-PCR handling of the samples. Besides it's clinical value, quantitative real-time PCR provides the opportunity to study the dynamics of respiratory virus infection in different patient population. It provides the possibility to assess virus load and duration of virus shedding and is useful to measure the effects of antiviral treatment.

In **Chapter 4** the development and validation of a quantitative multiplex real-time PCR for the simultaneous detection of influenza A and B viruses is described. We followed the dynamics of the infection in 6 patients showing a rapid decline in viral load in the first days of infection. Even though influenza virus infection usually only persists for one week, quantification is useful to evaluate the effects of antiviral therapy and to study the duration of viral shedding in hospitalized patients.

Compared with pneumonias caused by other respiratory viruses, RSV pneumonias are associated with the highest mortality in bone marrow transplant recipients and leukemia patients. Since immunocompromised patients and children are known to shed virus for a long period of time and RT-

PCR methods are found to be extremely sensitive, the clinical interpretation of a positive result is considered to be difficult. In the study described in **Chapter 5** we have analyzed control specimens by real-time PCR to exclude false-positive results and evaluated viral RNA detection shedding in immunocompromised adult patients during symptom free episodes. We did not find any clinically false-positive result for RSV in the specimens taken at set symptom-free moments and therefore conclude that this assay indeed has considerable diagnostic significance.

In **Chapter 6** we demonstrate the importance of real-time PCR for the identification of respiratory viruses that are difficult to identify otherwise. HCoV's are fastidious and their identification through culture is insensitive and laborious. We assumed that it is likely that as a result, the precise role of HCoVs in RTI's is much underestimated. Our findings demonstrate, that HCoV can indeed frequently be detected in clinical specimens received at the virology laboratory from patients with various presentations of RTI with the use of real-time PCR, and that the assay provides a useful tool for large scale epidemiological studies to further clarify the role of coronavirus infection in humans

Impact of respiratory viruses in specific patient populations

The development of new approaches for prevention and treatment of acute respiratory virus infection in patients at high risk requires studies on the etiology and severity of the infection with the use of sensitive detection methods. The results of the two studies described in **Chapters 7-8** emphasize that respiratory viruses should be recognized as important causes of severe lower respiratory tract disease in immunocompromised patients and that real time PCR is a clinically applicable tool for the diagnosis of virus associated pneumonia in immunocompromised patients.

In the retrospective study in adult hematological cancer patients hospitalized with pneumonia described in **chapter 7**, we show that molecular diagnostic techniques significantly increase the detection rate of respiratory viruses compared with traditional methods. The increased sensitivity is especially valuable to establish the involvement of HCoV and rhinoviruses in pneumonia in these vulnerable patients. We found an increased involvement of rhinoviruses in virus-associated pneumonia, indicating that rhinoviruses may play a serious role as a cause of pneumonia in immunocompromised patients. Surprisingly also HCoV was detected in one patient, an observation that has only been documented once previously in a severely compromised patient following autologous bone marrow transplantation for breast cancer. RSV was the most prevalent respiratory virus in respiratory virus associated pneumonia. The mortality rate of 20 per cent is consistent with previous studies, although some other groups have documented RSV pneumonia related mortality rates up to 83%. The retrospective character of the study restricted the interpretation of our results. This led us to conduct the prospective study described in **chapter 8** In this study we wanted to examine the frequency and severity of respiratory virus infection in recipients who underwent autologous or allogeneic stem cell transplantation (SCT) and to investigate the diagnostic value of real-time PCR for the detection of respiratory viruses as compared to viral culture. Our prospective

study showed an incidence of viral upper and lower respiratory tract infection of 14% with standard viral culture and of 36% with real-time PCR. This confirmed our previous observation that detection of respiratory virus infection by real-time-PCR is much more sensitive than with viral culture and antigen testing in recipients of SCT, especially in case of lower respiratory tract infection. In addition, we show that infections with respiratory viruses occurred frequently and were associated with severe lower respiratory tract infection. Rhinoviruses are the most frequent pathogen involved in upper respiratory tract infections. The role of Rhinovirus as the causative pathogen of pneumonia is not fully elucidated yet, but our results and those recently obtained by others strongly suggest their involvement in lower respiratory tract infection. In contrast to most other respiratory viruses, they could also be detected in 8% of the samples taken from patients without signs of RTI. The mortality associated with respiratory virus infection was low compared to other studies. Two patients died due to a viral pneumonia.

In **Chapter 9** we show with the use of quantitative real-time PCR that the clearance of respiratory virus in the upper respiratory tract is similar in asthmatic and non-asthmatic patients. Moreover, the maximum virus load and persistence in the URT preceded the asthmatic symptoms and was not associated with the persistence of asthmatic symptoms. The results of this study indicate that ongoing virus replication is not the explanation for the severity of asthmatic symptoms.

DISCUSSION AND FUTURE IMPLICATIONS

The etiology of about 25 % of upper respiratory tract infection and up to 50% of all patients with community-acquired pneumonia (CAP) remains uncertain [13,17,22]. Viral pathogens are important causes of acute respiratory tract infection, but with the current laboratory detection methods, the etiology remains undetermined in a significant number of patients with a presumed viral etiology for upper and lower respiratory tract infection. Moreover newly identified and emerging viruses will continue to change the etiology of viral RTI.

The unknown etiology of RTI can in part be attributed to the number of undiagnosed but known pathogens because of the lack of sensitivity of the currently used laboratory techniques. In this thesis the main focus was on respiratory viruses as etiologic agent for respiratory tract illnesses. In the recent years respiratory viruses have been recognized as important respiratory pathogens in immunocompromised patients [10,23,34-36]. We demonstrated that with the use of real-time PCR a significantly larger proportion of RTI in SCT recipients are associated with respiratory virus infection compared with current laboratory techniques such as virus culture and antigen detection. We also showed that with the use of real-time PCR a significant proportion of pneumonia in hematological cancer patients that were previously undetermined is associated with respiratory viruses. As was previously suggested, their role in severe pneumonia in hematological cancer patients has now become more evidence-based. Future research should focus on the role of respiratory viral infection in pneumonias in these and other immunocompromised patients, such as solid organ recipients and the

elderly with underlying medical conditions. Influenza viruses, RSV, adenoviruses and parainfluenza viruses are well known to be able to cause pneumonia [5,29]. However, their actual prevalence and impact is unknown. Large scale epidemiologic studies using molecular diagnostics for respiratory viruses are needed to further clarify their incidence and severity in pneumonia. The role of "the common cold viruses" rhinovirus and HCoV in pneumonia is not yet fully understood. Until recently, it was generally thought that rhinoviruses were not able to invade the lower respiratory tract. However, in vivo studies have shown that this is not the case and suggest that rhinovirus infections may be one of the most important causes of lower in addition to upper respiratory tract disease [16,26]. Questions that remain largely unanswered are: is it the primary pathogen, a contributing pathogen, or a pathogen predisposing to secondary infection with bacteria or fungi that then cause pneumonia or is sensitive detection with molecular methods reflecting contamination. With the use of real-time PCR there is no post-PCR handling of the samples, which makes the latter explanation highly improbable.

Other known respiratory pathogens (bacteria, fungi and nematodes) certainly account for a large proportion of the unknown etiology of RTI as well. Examples of respiratory pathogens that have regained interest are the atypical bacteria such as *chlamydia*, *mycoplasma*, and *legionella* species. With the use of more specialized tests for *legionella* spp., *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* their role becomes more evident as well. The incidence of *Legionella* infection ranges from 1% to 27% of CAP. Recent studies have found the prevalence of *M. pneumoniae* in adults with pneumonia to range from 1.9 to over 30%. *C. pneumoniae* account for 6-20% of CAP. These ranges depend on several factors such as setting of the studied population and age group examined but also depend largely on the diagnostic methods used [1,12]. PCR has proven to be at least as sensitive as culture in respiratory specimens. Quantitative real-time PCR for the sensitive diagnosis of these atypical bacteria will certainly be of great value as well if respiratory specimens can be obtained [6,15,18,21,28,32].

However, the remainder of unknown etiology in RTI is almost certainly due to the possibly large number of unidentified pathogens. Over the years we have seen several newly emerging or newly identified respiratory viruses. In 1993 a sudden, unexplained, and highly fatal respiratory illness of unknown etiology was identified and a previously unrecognized hantavirus species was held responsible for the outbreak of disease [30,37]. Since then other groups have reported occasional outbreaks of hantavirus pulmonary syndrome that often occur in clusters as a result of the epizoology of rodent hosts [7]. More recently, a new member of the paramyxoviridae family in the genera metapneumovirus was discovered and is held responsible for both upper and lower respiratory tract infection in both children and adults [2,11,33]. And finally, the latest outbreak of severe acute respiratory syndrome (SARS) has unraveled a newly identified coronavirus [8,20].

Besides previously unidentified respiratory viruses, newly emerging respiratory viruses pose a challenge for adequate laboratory diagnosis as well. Several reports have been published on avian influenza viruses such as the avian flu virus A/H7N7 and the Hongkong ckicken flu H5N1 that were

directly transmitted from birds to humans [4,14,31]. These assert a significant threat for new highly virulent reassortants that easily spread among humans and should be regarded as potential human pathogens that we have to take serious. Rapid diagnostic methods, such as PCR are then invaluable to limit the spread of disease. This was also demonstrated with the rapidity and ease at which a real-time PCR could be developed to detect new cases of SARS and has been used thereafter to identify new patients and control the outbreak [8,27].

The use of real-time quantitative PCR for the detection of respiratory viruses beholds more than a tool for rapid, specific and sensitive diagnosis. Quantifying viral RNA load facilitates the survey of viral dynamics in respiratory virus infection. By using real-time quantitative PCR in a small number of otherwise healthy patients infected with influenza virus A or B we showed that influenza could be detected up to 7 days after the onset of symptoms. In general, influenza virus A causes more severe infection compared to influenza virus B [38]. We did not document a clear difference in viral RNA load between influenza virus type A- and influenza virus type B-infected patients indicating that viral RNA load may not represent the pathogenicity of a virus. We also demonstrated that the viral persistence and RNA load in the upper respiratory tract of asthmatic patients neither reflected the symptoms of the lower respiratory tract nor the reduction in peak flow rate. In the studied population, symptoms were not related to virus load or virus shedding. The fact that the LRT symptoms in the asthmatic group were present for almost a week after the clearance of the virus indicates that there is an ongoing inflammatory response causing the asthmatic symptoms. Quantitative RT PCR to assess ongoing viral replication and disease progression in SARS infected patients was used in a recently published study [27]. No correlation between load, shedding and severity of symptoms was found in this prospective study and indicated that the damage is related to immunopathological damage as a result of overexuberant host response, rather than uncontrolled viral replication. We did however find a tendency towards prolonged shedding of respiratory viruses, in particular rhinovirus, in the studied population of SCT recipients. Prolonged shedding of respiratory viruses in the immunocompromised patient has been described previously [9,19]. It would be interesting to see whether this prolonged shedding and virus load reflects severity of infection, ongoing virus replication, and invasion of the lower respiratory tract. In contrast with other studies, we did not observe that SCT recipients with an URTI had progression to a LRTI [3,24,25,35]. All patients who were diagnosed with a LRTI were having clinical features of pneumonia without obvious complaints of a viral URTI, indicating that in immunocompromised patients respiratory viruses may cause pneumonia not preceded by an URTI. In the subgroup of SCT recipients with RSV related pneumonia we could not find a higher viral load compared to the patients were RSV was only confined to URTI. These groups however are too small to draw any conclusions.

Since several new antiviral agents against respiratory viruses are now available or under development, a rapid and sensitive method for detecting respiratory viruses is essential to implement immediate antiviral treatment and to prevent or limit spread of infection with respiratory viruses. The question is which groups to treat with antiviral agents or target for prophylaxis? In general, the incidence and severity of respiratory virus infection and related complications is especially high in the elderly, in persons with chronic underlying conditions and in immunocompromised patients. In these patients the protective effect of vaccination is limited. These patients would particularly benefit from accurate, early rapid diagnosis and subsequent adequate antiviral treatment, such as the novel neuraminidase inhibitors targeted against influenza viruses. Unfortunately, none of the neuraminidase inhibitors has so far been demonstrated to be effective in preventing i) severe influenza-related complications and ii) to be beneficial to severe immune compromised persons. Future research on the effectiveness of treatment with new antiviral agents should focus on their use in these groups. Quantitative real-time PCR is a useful tool to assess the effects of antiviral treatment. Prophylactic administration should be considered in at-risk patients in closed communities, such as nursing homes or hospitals, when vaccination provides inadequate protection. A similar situation has recently been actualized by an attempt to limit the spread of chicken flu among humans with the use of antiviral agents [31]. It is doubtful whether antiviral treatment targeted against ongoing replication of respiratory viruses will be worthwhile in otherwise healthy adults. It appears that viral load declines rapidly in these patients and therefore treatment must be started in a very early phase of the infection in order to be effective. In the asthmatic group studied in this thesis we showed that the persistence of the virus is not associated with the persistence of asthmatic symptoms. Therefore, it is also questionable whether anti-viral therapy will be effective after the onset of an exacerbation. It would be interesting to further elucidate the inflammatory response of respiratory virus's infections. This may lead to more effective drugs that modulate the inflammatory events following virus infection.

Finally, the value of real-time PCR in terms of speed, sensitivity and specificity are clearly shown. More standardized protocols that are independently evaluated by different laboratories will first need to be established before PCR can become routine diagnostics for respiratory viruses. In practice it can attribute to limit the use of antibiotics and apply appropriate treatment and be valuable in the early detection of hospital outbreaks of community respiratory viruses. However, precise and detailed information on the causative agents of RTI, as well as on the pathogenicity and impact of respiratory viruses will be needed to convince physicians to alter the habits in prescribing antibiotics. Only then, the clinical value and cost effectiveness of real-time PCR can be further addressed.

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Chapter 11

Samenvatting

Van oorsprong heeft de nadruk van de preventie en behandeling van respiratoire virussen altijd gelegen op influenza virus infectie (de "griep"). De epidemiologie, impact en ernst van influenza is uitgebreid onderzocht en het is overtuigend aangetoond dat influenza elk jaar zorgt voor aanzienlijke ziekte en sterfte onder de bevolking. Ondanks deze associatie wordt influenza, net als andere respiratoire virus infecties vaak onterecht beschouwd als een onschuldig verkoudheidsvirus en behandeling en preventie niet nodig geacht. Antivirale middelen tegen influenza worden dan ook nauwelijks voorgeschreven. Het gebruik van de al langer bestaande middelen tegen influenza is tevens beperkt vanwege de aanzienlijke bijwerkingen en het snel ontstaan van influenza virussen die ongevoelig zijn voor deze middelen. Recent is er een nieuwe klasse antivirale middelen ontwikkelt, met een ander werkingsmechanisme, de zogenaamde neuraminidase remmers. Een overzicht van klinische studies wordt beschreven in **hoofdstuk 2**. Tijdige behandeling met een neuraminidase remmer verkort de ziekteduur met ongeveer anderhalve dag in anderszins gezonde individuen. Naast het verkorten van de ziektelast zou dit economisch gunstig kunnen zijn voor de werkende bevolking. De therapeutische toepassing van de middelen kent echter ook nadelen. Zo moeten ze in een vroeg stadium van de infectie worden toegediend om effectief te zijn. Griepachtige ziektebeelden worden echter niet alleen veroorzaakt door het influenza virus maar kunnen ook veroorzaakt worden door tal van andere respiratoire virussen die gelijksoortige klachten geven. Daarnaast zijn er ook verschillende bacteriële verwekkers die hetzelfde griepbeeld kunnen geven. De antivirale middelen werken niet tegen deze andere verwekkers. Momenteel bestaat er echter nog geen snelle, gevoelige en betrouwbare test om tot een diagnose te komen. In de huisartsenpraktijk, die met name in aanraking komt met de gezonde individuen met griepachtige klachten, zorgt dit voor een dilemma: wie nu wel en wie nu niet te behandelen als je niet weet wat de verwekker is. In **hoofdstuk 3** hebben we onderzocht of er toch niet typische klachten zijn die passen bij een infectie met het influenza virus. Hiervoor zijn al verschillende nationale en internationale criteria opgesteld. In de beschreven studie populatie was het onderscheidend vermogen van beide criteria zeer beperkt. De best voorspellend waarde, van 75%, bestond uit de klachten combinatie hoesten, koorts en hoofdpijn tijdens de jaarlijkse griepepidemie bij patiënten die niet gevaccineerd waren. Geconcludeerd wordt dat het tijdig opsporen van de griepepidemie door middel van actieve surveillance en het berichten van de huisartsen een belangrijk aandeel hebben in het juist diagnosticeren van influenza. Niettemin lijkt snel en gevoelig laboratorium onderzoek toch een vereiste voor effectief en efficiënt voorschrijven van eventuele antivirale therapie. De conventionele laboratorium methoden voor het detecteren van respiratoire virussen zijn of arbeid- en tijdsintensief en/of niet specifiek en gevoelig genoeg.

In **hoofdstuk 4-6** tonen we aan dat met behulp van de ontwikkelde en hier beschreven detectiemethoden op basis van de real-time polymerase keten reactie (PCR) we op snelle, gevoelige en specifieke wijze influenzavirussen, respiratoir syncytieel virus (RSV) en coronavirussen (HCoV) kunnen aantonen. De beschreven hoofdstukken laten zien dat deze methoden een aanzienlijke verbetering zijn ten opzichte van de bestaande conventionele detectie methoden zoals viruskweek en

virussnelkweek op basis van antigeendetectie. Met behulp van de real-time PCR kan binnen 5 uur de verwekker worden aangetoond. Bovendien is de techniek, omdat de reactie in een gesloten systeem plaatsvindt, niet gevoelig voor contaminatie en kan dus ook goed buiten een onderzoeks-setting worden gebruikt. Met de techniek kan ook de hoeveelheid virus worden aangetoond. Dit kan goed gebruikt worden om de dynamiek van een virusinfectie en het beloop in de tijd te volgen. Zo kan worden gevolgd of de infectie nog aanwezig is en of een therapie aanslaat. Met name coronavirussen zijn moeilijk te detecteren door middel van viruskweek, en het aandeel van deze virussen is dus ook nog niet goed opgehelderd. Met behulp van de real-time PCR vonden we dat een groot deel (11%) van de patiënten met klachten van een luchtweginfectie het coronavirus met zich meedragen.

Met name patiënten met een verminderde weerstand of een chronische ziekte hebben de kans om ernstig ziek te worden van de "gewone" respiratoire virussen. Om nieuwe antivirale middelen toe te passen op deze patiënten en er zorg voor te dragen dat goede preventieve maatregelen kunnen worden getroffen om infectieverspreiding tegen te gaan is het eerst nodig om met adequate en gevoelige detectiemethoden het aandeel van de verschillende respiratoire virussen in het ontstaan van ernstige longontsteking en complicaties te onderzoeken. In **hoofdstuk 7** beschrijven we hoe retrospectief in patiënten met een hematologische maligniteit en een pneumonie met behulp van de PCR detectie methoden in 35% van de patiënten een respiratoir virus wordt aangetoond in tegenstelling tot de conventionele methoden waarmee slechts in 19% van de patiënten een virus wordt aangetoond. In 21% van de patiënten wordt dit respiratoire virus als enige mogelijke verwekker gevonden. Dit ogenschijnlijk aanzienlijk aandeel leidde tot de studie beschreven in **hoofdstuk 8**. In deze prospectieve studie wordt het voorkomen en de ernst van luchtweginfecties geassocieerd met een respiratoir virus onderzocht in patiënten die een beenmergtransplantatie ondergingen. Opnieuw wordt in 36 % van de luchtweg infecties een respiratoir virus aangetoond met behulp van de real-time PCR methode. Met name het gewone verkoudheidsvirus, rhinovirus, wordt vaak aangetoond, zowel bij bovenste als bij onderste luchtweginfecties. Hoewel de respiratoire virussen vaak werden aangetoond was het beloop van de infecties over het algemeen mild. Twee patiënten overleden met grote waarschijnlijkheid ten gevolge van de respiratoir virusinfectie.

Een astma aanval wordt vaak vooraf gegaan door een verkoudheid. In **hoofdstuk 9** wordt het beloop van een respiratoire virusinfectie in gezonden vergeleken met het beloop in patiënten met astma. Zowel de hoeveelheid virus als de duur van virus uitscheiding is gelijk in beide groepen. De astma patiënten ondervinden echter significant meer klachten, met name astmatische klachten. Dit zou kunnen betekenen dat andere factoren (zoals bijvoorbeeld een ontremde ontstekingsreactie) dan de virusinfectie zelf aanleiding zijn tot het ontstaan van de klachten en de virusinfectie alleen een aanzet geeft.

Tenslotte worden in **hoofdstuk 10** de resultaten samengevat en besproken. De laatste jaren is veel aandacht besteedt aan de rol van respiratoire virussen, m.n. in patiënten met een gestoorde afweer. Met behulp van gevoelige detectiemethoden zien we dat deze virussen veelvuldig worden aangetoond en lijkt het erop dat respiratoire virussen ondergedetecteerd zijn geweest. Daarnaast is er veel aandacht voor gevoelige detectie methoden voor snelle diagnose van nieuw ontstane respiratoire virus varianten (HongKong "kippe-griep", SARS). Gezien de snelle ontwikkeling in behandeling van respiratoire virussen en vaccin ontwikkeling , zullen er in de toekomst mogelijk ook betere interventie methoden zijn. Toekomstig onderzoek naar de precieze rol en pathogeniciteit van de verschillende virussen in verschillend patiënt populaties zal de klinisch aanvullende waarde van real-time PCR moeten aantonen.

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CURRICULUM VITAE

Leontine van Elden werd op 4 juni 1969 geboren te Bunnik. Na in 1987 het VWO diploma gehaald te hebben op het Marnix College te Ede, studeerde ze een jaar aan de Springbank Community Highschool, te Calgary, Canada. Vervolgens werd in 1988 gestart met de studie geneeskunde aan de Universiteit Utrecht. In 1992 vertrok zij voor 4 maanden naar El Kef, Tunesië, om een veld studie te doen ter evaluatie van open hartchirurgie van tunesische kinderen in Nederland. In 1994 werd het doctoraal examen gehaald. Eind 1995 werden gedurende 5 maanden klinische stages gelopen in Zuidelijk Afrika: dermatologie en traumatologie in het Tygerberg Hospitaal (universiteit van Stellenbosch, Zuid Afrika), gynaecologie & verloskunde in Bulawayo United Hospitals (Zimbabwe). In 1997 werd het arts-examen behaald. Na een half jaar als arts-assistent longgeneeskunde gewerkt te hebben in Medisch Centrum Alkmaar, werd in 1998 gestart op de afdeling virologie van het Universitair Medisch Centrum Utrecht, met het onderzoek wat geleid heeft tot dit proefschrift (promotor Prof. dr. A.I.M. Hoepelman). Op 1 januari 2001 is zij begonnen met de opleiding interne geneeskunde in het Meander Medisch Centrum, te Amersfoort. In april 2003 is zij gestart met de opleiding Longziekten & Tuberculose in het Universitair Medisch Centrum Utrecht (opleider Prof. dr. J-W. J Lammers).

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