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Article

Epidemiological Investigation of Nine Respiratory Pathogens in Hospitalized Children in Germany Using Multiplex Reverse-Transcriptase Polymerase Chain Reaction

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Abstract The aim of this study was to generate urgently needed data on respiratory pathogens in German children using an economical and efficient tool. Nasopharyngeal aspirates of hospitalized children 0–16 years of age with an acute respiratory tract infection were tested by a nine-valent multiplex reverse-transcriptase polymerase chain reaction. Of 1281 children, 449 (35%) had an acute respiratory tract infection caused by at least one of the organisms studied; there were 29 cases of dual infection. At least 34–42% of severe acute respiratory tract infections in children under 5 years of age were caused by viruses. In children over 5 years of age, this proportion was 23% ($P<0.001$). Infection during the first 2 years of life was most frequently due to respiratory syncytial virus ($n=162$ cases). Parainfluenza virus type 3 ($n=22$) and type 1 ($n=14$) were detected almost exclusively in children under 5 years of age. Influenza A ($n=90$) and adenoviruses ($n=98$) were prevalent in all age groups. The frequency of influenza B virus isolation ($n=17$) rose significantly after the age of 5 years. *Mycoplasma pneumoniae* infection ($n=24$ cases, 5.2%) was most frequent in 5- to 16-year-old patients. Only one case of *Chlamydia pneumoniae* infection was found. Since the distribution of pathogens within the different types of lower respiratory tract infections is very similar, it seems that host factors determine which form of lower respiratory tract infection develops in an individual patient. The multiplex reverse-transcriptase polymerase chain reaction may, in the future, become an important tool for epidemiological studies as well as for individual diagnosis.

Introduction

Acute respiratory tract infections (ARTIs) are the primary cause of childhood morbidity (in both developed and developing countries) and mortality (in developing countries). Epidemiological research is fundamental in elucidating the behavior of the different organisms involved and their impact on the burden of disease. Obtaining epidemiologicial data is a prerequisite for focusing further research and vaccine development, for the design of interventional studies and for the development of prevention and treatment strategies. Such data are urgently needed as new tools for treatment and prevention of these infections appear on the horizon. We conducted a hospital-based study to analyze severe forms of ARTI. A nine-valent multiplex reverse-transcriptase polymerase chain reaction was developed in our unit as a diagnostic tool for this purpose. This technique should also enable us to serve peripheral hospitals and private practices that do not have access to a highly sophisticated virological laboratory. Since it was designed for nasopharyngeal aspirates, organisms that usually do not colonize the upper respiratory tract were chosen for the panel. Other factors considered when selecting the pathogens were the data on ARTI pathogens and the appropriate primers available at the time this technique was being developed (1995).

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Materials and Methods

From December 1995 to March 1999, nasopharyngeal aspirates from children 0–16 years of age who were seen in one of the pediatric hospitals in Kiel (University Children's Hospital or Municipal Hospital) were tested using multiplex RT-PCR as described recently [1]. Kiel is the capital of Schleswig-Holstein, the northernmost federal state of Germany. In 1995 the total population of Schleswig-Holstein was 2.7 million, of whom 427,516 (15.7%) were children under 16 years of age [2]. The 1995 birth cohort was 27,430 newborns. The University Children's Hospital Kiel (112 beds) serves the city of Kiel (237,030 inhabitants) as well as the western and northern parts of Schleswig-Holstein. For the latter, however, it mainly functions as a tertiary referral hospital and admits about 4600 children per year. The Municipal Hospital's Department of Pediatrics (52 beds) mainly serves the city of Kiel and nearby areas and admits an average of 2800 children per year.

The inclusion criterion was any ARTI, regardless of the reason for hospital admission. The nasopharyngeal aspirates and basic data were collected prospectively. The final diagnosis, which was used for categorization of cases into disease entities, was obtained from the discharge letter. The diagnosis was made on the basis of the chest radiograph and clinical findings. As wheezing is of major interest in pediatrics and can influence treatment, pneumonia as a disease entity was divided into two groups: pneumonia with wheezing and pneumonia without wheezing. Cases diagnosed as bronchiolitis were added to the group "wheezing bronchitis", since the definition and basic concepts of this entity lack uniformity [3]. The nasopharyngeal samples were obtained within 48 h of admission, if possible, and processed immediately or stored at 4 °C until the next working day.

The multiplex RT-PCR used has been described in detail previously [1]. Briefly, before July 1997, nucleic acid preparation was done by phenol-chloroform extraction and ethanol precipitation. Thereafter, the Boehringer-Mannheim High Pure Viral Nucleic Acid Kit was used (Boehringer Mannheim, Germany). The multiplex RT-PCR involved the reverse transcription of the RNA from RNA organisms (respiratory syncytial virus [RSV], parainfluenza virus type 1 [PIV-1] and type 3 [PIV-3], influenza A virus, influenza B virus and enterovirus), followed by PCR amplification of the corresponding cDNA and of the DNA of adenovirus, *Mycoplasma pneumoniae* and *Chlamydia pneumoniae*, respectively. Primers were chosen from previously published highly conserved target sequences. PCR products were analyzed on 2% agarose gels and specified using the PCR-ELISA-Dig Detection System (Roche Diagnostics, Germany).

The data were stored and processed in a computerized database (Microsoft Access; Microsoft, USA). For statistical analyses, the one-tailed chi-squared test with one degree of freedom and the two-tailed Student's *t* test for small sample sizes were applied.

Results

From December 1995 to March 1999, nasopharyngeal aspirates from 1281 patients were received. The case ascertainment increased over the study period and reached more than 95% after the first year (total average 82%). Of the children diagnosed, 449 (35%) had an infection with at least one of the nine organisms investigated; 29 (6.5%) of these 449 had a dual infection. All but one dual infection included adenovirus or enterovirus. Respiratory syncytial virus (RSV) was detected along with influenza B virus in only one case. Thus, a total of 478 PCR-positive findings ("PCR isolates") were obtained. Table 1 presents a summary of the results.

Age Distribution. In the younger age groups 41% of the infected children were infants (Table 1). Among children up to 5 years of age, 34–42% were infected with one of the nine organisms, and among school-age children only 22.6% ($P < 0.001$) had a positive PCR result.

Pathogen Distribution and Patient Age. In the first 3 months of life, RSV was the most common pathogen detected, infecting 30% of the children (Table 1). For infants aged 4 months to 1 year, RSV (18.7%), adenovirus (8.9%) and influenza A virus (6.8%) were the most frequent organisms. Interestingly, one-fourth of all *Mycoplasma pneumoniae* cases occurred in this age group. With increasing age, the frequency of RSV among the nine organisms under investigation declined and that of adenovirus, enterovirus and influenza A virus rose. Infection due to parainfluenza virus type 3 (PIV-3) was similar clinically to RSV infection in the younger age groups but was far less frequent. Parainfluenza virus type 1 (PIV-1) appears to be predominant in toddlers and preschool children. In school-age children and adolescents, influenza A and B viruses, adenovirus and *Mycoplasma pneumoniae* were the major organisms causing severe ARTI.

Pathogen Distribution and Season. The seasonal distribution of the nine organisms is shown in Figure 1. RSV and influenza A virus infections occurred predominantly in winter. RSV infections began to occur mainly in December and were followed by influenza A infections in late winter. The 1997/1998 RSV season was prolonged, and the 1998/1999 season began earlier than expected. For the other pathogens, no clear seasonal trends were observed.

Distribution of Disease Entities and Pathogens. As this study was hospital-based, lower respiratory tract infections (LRTIs) were diagnosed more often than upper respiratory tract infections (URTIs) (Figure 2). Influenza A virus, influenza B virus and adenovirus were detected most often in patients with upper respiratory tract symptoms. The frequency of positive PCR results was highest ($\sim 50\%$) in the disease groups with wheezing (Figure 3).

The relative proportion of the viral pathogens was very similar for all types of LRTI (Figure 4). In cases of infection with *Mycoplasma pneumoniae*, wheezing was predominantly absent. In 27 cases of otitis media, adenovirus was found in eight and RSV and influenza A virus in seven cases each, respectively.

Respiratory Syncytial Virus. RSV was detected in 162 nasopharygeal specimens (12.7% of the total sample,

Table 1 Pathogens detected by multiplex reverse-transcriptase polymerase chain reaction (m-RT-PCR) in each age group

Pathogen	No. (%)					
	0–3 months	4–12 months	1–2 years	2–5 years	5–16 years	Total
RSV	57 (30.0)	63 (18.7)	22 (9.4)	18 (7.2)	2 (0.7)	162 (12.7)
PIV-3	5 (2.6)	8 (2.4)	4 (1.7)	4 (1.6)	1 (0.4)	22 (1.7)
PIV-1	0	4 (1.2)	6 (2.6)	4 (1.6)	0	14 (1.1)
Influenza A	5 (2.6)	23 (6.8)	16 (6.8)	28 (11.3)	18 (6.7)	90 (7.0)
Influenza B	2 (1.1)	3 (0.9)	3 (1.3)	2 (0.8)	7 (2.6)	17 (1.3)
Adenovirus	8 (4.2)	30 (8.9)	22 (9.4)	21 (8.4)	17 (6.3)	98 (7.7)
Enterovirus	7 (3.7)	14 (4.2)	12 (5.1)	14 (5.6)	3 (1.1)	50 (3.9)
M. pneumoniae	1 (0.5)	6 (1.8)	0	3 (1.2)	14 (5.2)	24 (1.9)
C. pneumoniae	0	0	0	0	1 (0.4)	1 (0.1)
Total[a]	85	151	85	94	63	478
Total positive[b]	80 (42.1)	138 (41.0)	80 (34.0)	90 (36.1)	61 (22.6)	449 (35.1)
Total negative[c]	110 (57.9)	199 (59.0)	155 (66.0)	159 (63.9)	209 (77.4)	832 (64.9)
Total[d]	190 (100.0)	337 (100.0)	235 (100.0)	249 (100.0)	270 (100.0)	1281 (100.0)

[a] Total number of PCR isolates
[b] Number of patients with at least one isolate detected by m-RT-PCR
[c] Number of patients with m-RT-PCR negative findings
[d] Total number of patients per age group
RSV, repiratory syncytial virus; PIV-3, parainfluenza virus type 3; PIV-1, parainfluenza virus type 1

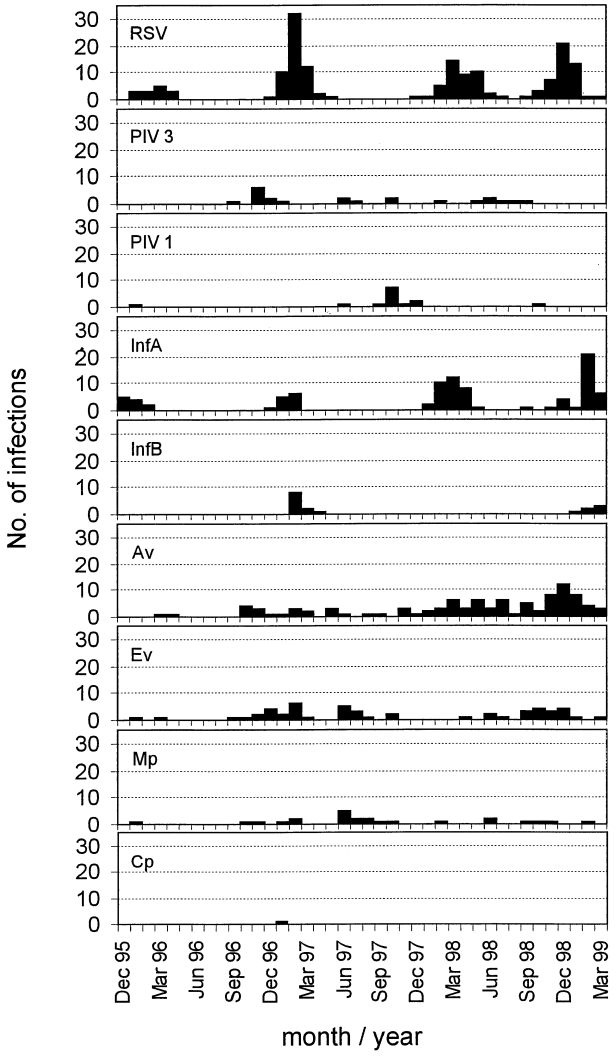

Figure 1 Seasonal occurrence of pathogens in absolute numbers of infections over time

34% of all positive results). In the age group 0–3 months, 57 of 190 patients (30% of this age group, 67% of all positive results; Table 1) were positive for RSV. RSV was transmitted nosocomially on 14 occasions. RSV caused LRTI in 149 of the 162 cases; 80 of these patients presented with wheezing.

RSV infections occurred in yearly epidemics. In the region studied, the RSV season usually starts in December and ends in April (Figure 1). The 1997/1998 outbreak, however, extended into July 1998, and the following outbreak began early, in September 1998, bringing the two outbreaks closer together.

Parainfluenza Virus Type 3. PIV-3 was detected in 22 cases (1.7% of the total sample, 4.6% of the positive results). Clinically, PIV-3 disease was similar to RSV infection in children under 2 years of age, but it occurred much less frequently (10–25% as frequent as RSV; Table 1). In toddlers and preschool children, it was mainly involved in cases of laryngotracheobronchitis (LTB). No distinct seasonal pattern or outbreaks were seen.

Parainfluenza Virus Type 1. PIV-1 infection was detected in 14 cases (1.1% of the total sample, 2.9% of positive results), mainly in the autumn and early winter 1997. All cases occurred in children aged 4 months to 5 years. PIV-1 was found in six (16%) cases of LTB (Figure 4). Interestingly, in only 23 of 61 cases of LTB could any of the seven viruses investigated be detected. PIV-1 was involved in only one case with alveolar infiltration.

Adenovirus. Adenovirus infection was diagnosed in 98 cases (7.7% of the total sample, 20.5% of positive results), 16 of which were dual infections. It was detected together with RSV in eight of 162 cases, with

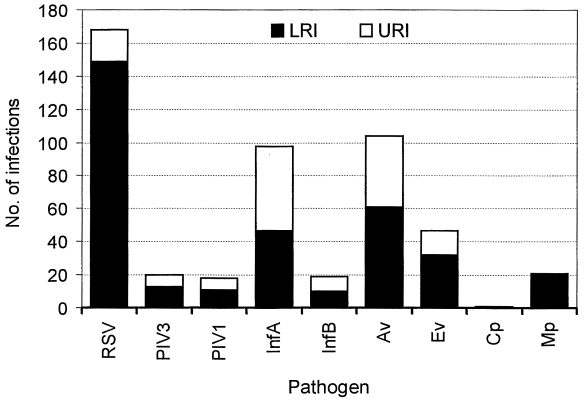

Figure 2 Proportion of upper respiratory tract infections versus lower respiratory tract infections per causative pathogen

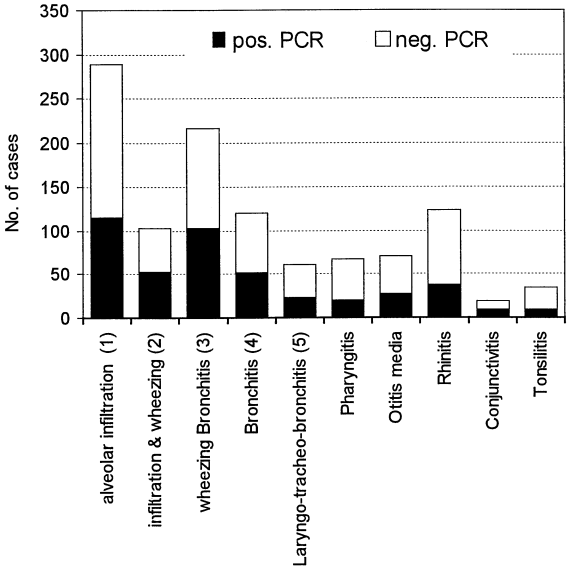

Figure 3 Disease entity and PCR results in absolute numbers (1) pneumonia; (2) pneumonia with wheezing; (3) obstruction of small airways; (4) no wheeze, no infiltrates; (5) laryngotracheobronchitis (LTB) (obstruction of large airways) (bronchiolitis included in wheezing bronchitis)

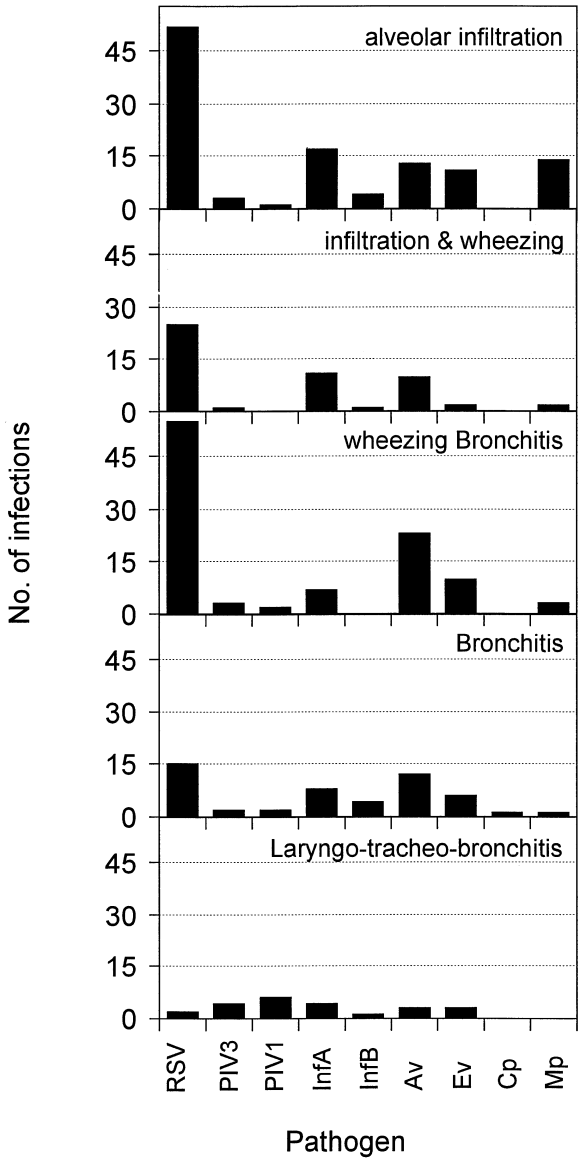

Figure 4 Lower respiratory tract disease entities and pathogen distribution in absolute numbers (bronchiolitis included in wheezing bronchitis)

influenza A virus in two of 90 cases and with enterovirus in four of 50 cases. This correlates well with the prevalence of these three organisms within the total sample. During most of 1998, the incidence of adenovirus infection was higher than that observed previously, most markedly in late autumn. Five of nine patients with distinct conjunctivitis were diagnosed with an adenoviral infection. The frequency of adenovirus infection rose continuously with age, peaking in children over 5 years of age (Table 1). A total of 38 (39%) adenoviral infections occurred in infants. Of the 98 adenovirus-positive patients, 61 (62%) had an LRTI. Thirty-three (54%) of these 61 patients presented with wheezing. Acute otitis media was diagnosed together with adenoviral infection in eight (8%) cases.

Enterovirus. Enterovirus ($n=50$ cases, 3.9% of the total sample, 10.5% of positive results) was the most frequent cause of ARTI in toddlers and preschool children. Within these age groups, up to 15% of severe ARTIs involved enterovirus. Sixteen (32%) of the 50 cases occurred as dual infections with RSV, influenza A virus or adenovirus. In three cases enterovirus was detected together with PIV-3 during autumn 1996, summer 1997 and 1998, when the peak seasons of the two organisms coincided. Most cases occurred in summer and autumn, but there was also a high incidence in winter and spring 1996 (Figure 1).

Influenza A Virus. Influenza A virus was found in 90 cases (7% of the total sample, 18.8% of positive

results). The incidence of 2.6% in early infancy rose to 6.8% in later infancy and remained high thereafter, reaching a peak in preschool children (11.3%, Table 1). Influenza A virus caused the highest proportion of URTIs , and many of the URTIs and LRTIs due to this virus occurred simultaneously (Figure 2). There was no preponderance of any particular type of LRTI due to influenza A virus. Influenza A epidemics occurred yearly in late winter. Like the RSV epidemics, the interval between the influenza A epidemics of 1997/98 and 1998/99 was somewhat shorter than that in previous years.

Influenza B Virus. Influenza B virus infection was detected in only 17 cases (1.3% of the total sample, 3.6% of positive results). It was found in 2.6% of school-age children and adolescents. Only one patient presented with wheezing. A 2-year interval was observed between the two outbreaks, which occurred in winter 1996/97 and winter 1998/99.

Mycoplasma pneumoniae. The occurrence of *Mycoplasma pneumoniae* infection ($n=24$ cases, 1.9% of total sample, 5% of positive results) appears to have a biphasic distribution with regard to age, peaking first in infants and again in children over 5 years (Table 1). Of note is that *Mycoplasma pneumoniae* does not seem to be involved in URTIs. Furthermore, wheezing was present in only four of 24 cases of infection. The mean age in these four cases (3.9 years) was not significantly lower than that in the 20 nonwheezing cases (6.6 years). No outbreak of *Mycoplasma pneumoniae* infection has been observed thus far.

Chlamydia pneumoniae. Chlamydia pneumoniae was detected in only one child, in association with adenovirus. The 7-year-old patient with bronchitis presented with a 2-week history of cough as well as signs of conjunctivitis and rales on admission.

Discussion

Using a multiplex RT-PCR, an epidemiological investigation of nine supposedly noncolonizing pathogens in hospitalized children was conducted over a time period of 40 months, including four winter seasons. After the first year of the study, nasopharyngeal aspirates from more than 95% of all ARTIs were tested by multiplex RT-PCR. If a pathogen was identified from the nasopharyngeal aspirate of a child with a concurrent LRTI, a causal relationship to the LRTI was assumed as long as no upper airway colonization known for the organism found. We are aware that these assumptions have limitations and may have to be modified after new data become available, but invasive techniques such as lung puncture, bronchoscopy and paired sera testing are not feasible for diagnosis of LRTIs in Germany. Blood cultures are generally of no help in diagnosing LRTI in children.

The nasopharyngeal aspirate is regarded as the appropriate specimen from which to recover noncolonizing pathogens. As Ruuskanen [4] pointed out, the rate of recovery of viruses from nasopharyngeal aspirates is 20–30% higher than that from nasal swabs and 50% higher than that from throat swabs. For *Chlamydia pneumoniae*, this might be different because it is an intracellular organism. Since culture is still regarded as the gold standard for isolation of respiratory viruses other than RSV (for which indirect immunofluorescence assay is recommended), the use of the multiplex RT-PCR challenges this method as a tool for epidemiological investigations. Despite "screening type" capabilities, if several cell lines are used simultaneously, viral cultures require viable organisms and a highly sophisticated laboratory associated with maintenance of costly and vulnerable cell lines. RSV seems to be especially vulnerable so that any outreach epidemiological investigation may easily be biased towards a false low incidence of RSV infection. Beyond this, several of the organisms of interest are difficult to culture, e.g. *Chlamydia pneumoniae* and *Mycoplasma pneumoniae*. The speed of culture in contrast to PCR is also slower, even when early detection systems such as centrifugation-amplified cultures (shell vial or spin cultures) are used [5]. Most markedly, this applies to rhinovirus (not yet included in the 9-valent multiplex RT-PCR), which requires up to 28 days to appear in cell culture [6, 7]. The multiplex RT-PCR requires 24–48 h of laboratory time, the same as that required for immunofluorescence tests, if performed on large sample numbers, but more than that required for rapid enzyme immunoassays (EIAs). However, to date, commercial EIAs are available only for RSV and influenza A virus. An important argument in favor of culture techniques is that further analyses can be performed on the isolates. Even this advantage can be matched by multiplex RT-PCR to a certain extent. Since the prepared nucleic acid can be stored and the PCR repeated, further studies of the amplicons can be performed. Determination of nucleic acid sequences, for example, can be used for molecular epidemiology, subtype analysis or tracing of mutations. A further argument strongly in favor of multiplex RT-PCR is cost. The spectrum of organisms included in this nine-valent multiplex RT-PCR would cost roughly USD 700 to test by conventional culture [4]. The cost for the multiplex RT-PCR is USD 55, plus labor.

As indicated by our sampling of hospital admissions, patients in the younger age groups were predominant, as is generally reported [8]. However, susceptibility to viruses with a high number of subtypes, such as adenovirus and enterovirus, extends into older age groups. Influenza viruses change by antigenic shift and drift. We call this causation the "subtype phenomenon", since in addition to the R_o (force of infection) and immunity, the number of subtypes seems to determine at what age a certain type of pathogen prevails.

The frequency of viruses as a cause of severe ARTI generally declines with age. As shown in this study, 35–40% of severe ARTIs requiring hospitalization in children under 5 years of age can be related to viruses. The proportion of viral infections among severe ARTIs can be even higher, according to data presented by Ruuskanen [4], who showed that rhinovirus can cause 20–24% of LRTIs. Furthermore, there are still a considerable number of pathogens not included in the nine-valent multiplex RT-PCR. In school-age children and adolescents, viruses occur significantly less frequently as a cause of severe ARTI ($P < 0.001$).

Epidemics due to RSV and influenza A virus occurred yearly. The interepidemic interval for the remaining organisms might well be longer than the 3-year observation period of this study. As in most countries worldwide, RSV epidemics occurred yearly. RSV is particularly prevalent in infants below the age of 4 months. In this study, 57 of the 190 infants in this age group were infected with RSV. Berner et al. [9] made a similar observation in a German pediatric population. The summer of 1998 was extremely cold and wet in northern Germany, which seems to explain why the 1997/1998 epidemic extended into July and the 1998/1999 epidemic began prematurely, in September 1998. Similar observations were made earlier by Carlsen et al. [10] in Oslo, Norway, and by Florman and McLaren [11] in Albuquerque, New Mexico, USA. In our study, more than 50% of patients with LRTIs due to RSV presented with wheezing. Otherwise, the number of cases of LRTI with alveolar infiltrates on the chest radiograph was equal to that with no infiltrates.

The clinical presentation of PIV-3 LRTI is very similar to that of RSV LRTI [12]. In this study PIV-3 infection occurred in only 10–25% as many children as RSV, increasing with age. PIV-3 infection occurs earlier in life than PIV-1 infection [13]. Glezen et al. [14] reported a higher incidence of PIV-3 infection in Houston, Texas, USA, with epidemics occurring seasonally in late winter to spring of each year. In the study presented here and the one by Carlsen et al. [10], the incidence of PIV-3 infection was lower, and no marked seasonal trends were observed. This discrepancy might be explained by geographical differences between Houston and Kiel or Oslo, respectively.

As most of the 14 cases of PIV-1 infection occurred in autumn 1997, an as-yet-unknown multiyear interepidemic period can be presumed, though a period of more than 1 year can be expected. Data from the 1999/2000 winter season will show whether a 2-year cycle, as described by Glezen et al. [14], can be confirmed (evidence of a 2-year cycle was confirmed in autumn 1999; unpublished data). Interestingly, only 61 (4.8%) patients had LTB, 23 of whom were positive for one of the nine pathogens. This indicates the presence of other pathogens such as PIV-2, which is not yet included in the multiplex RT-PCR we used. Since Kiel and Schleswig-Holstein have a maritime climate and good air quality, LTB and other related viruses, seem to be less prevalent.

Adenovirus was the second most prevalent pathogen studied (7.7%). This confirms data of Carlsen et al. [10] from Oslo. As adenoviruses can be shed over an extended period of time [15], there is a higher probability of coincidence with other pathogens and several disease entities. Therefore, their impact can be easily overstated. To date, 42 adenovirus subtypes are known, of which about nine are known to cause ARTI (types 1, 2, 3, 4, 5, 6, 7, 14 and 21) [15]. No analysis of these subtypes was done in the present study, as adenovirus general does not occur seasonally [16, 17]. We found the incidence of LRTI due to adenovirus to be 62%, considerably higher than the 9% and 27% described earlier by Ruuskanen et al. [16] and van Lierde et al. [18], respectively. This difference may be attributable to different study designs.

At least 27 of the 68 non-polio, non-hepatitis A enteroviruses should be detectable by the primers used in the multiplex RT-PCR [1, 19, 20]. Most enteroviral infections are asymptomatic or cause only minor illness [21]. As the duration of infection can be longer than that for other viruses, the likelihood of concurrent infections and overestimation of the related burden of disease is increased. In general, enteroviral-associated ARTI is considered a disease of infants and young children up to 5 years of age [21, 22], as observed in the present study. Although enteroviral infections in general are known to occur more frequently during the summer-autumn season [21], no clear such seasonality was observed for entroviral ARTIs.

The incidence of influenza A virus appears low in early infancy but high thereafter. The incidence seems to peak in preschool and school-age children. This is probably related to the increased risk among children of this age to encounter a contagious person. Glezen [23] even calls this age group the “fire of the epidemic”. The sharp rise in incidence after 3 months of age is most likely caused by waning maternal antibodies [24].

Influenza B epidemics are known to occur in approximately 3- to 4-year cycles [25]. Infection prevails in older children and rarely causes severe disease leading to hospitalization. Two reasons might explain why the incidence of influenza B infection in this study was not higher. First, the time interval studied may have been within an interepidemic phase. Secondly, as our study was hospital-based, we simply did not encounter considerable numbers of severely sick children with influenza B virus infection. The National Influenza Sentinel Network in Germany, however, reported that there was a considerable nationwide outbreak of influenza B infection in late winter 1999 [26].

Mycoplasma pneumoniae outbreaks have been reported to occur in 4- to 7-year cycles [27]. Therefore, we might not have yet encountered an epidemic with *Mycoplasma pneumoniae* in our region. Interestingly, however, in the neighboring country of Denmark, a *Mycoplasma pneumoniae* epidemic that occurred in winter 1998/1999 [28] was not observed in adjacent Schleswig-Holstein, the area we studied. Pneumonia without wheezing was the most common presentation of *Mycoplasma pneumoniae* infection, a finding also observed by Gendrel et al. [29]. Whether this is related to the pathogen itself or to the age of the patient remains unclear. The four patients with wheezing were not significantly younger than the children without wheezing (3.9 vs. 6.6 years). It seems worthwhile to test on a larger sample the hypothesis of age as a key variable associated with wheezing in general and with *Mycoplasma pneumoniae* infection in particular.

The prevalence of *Chlamydia pneumoniae* infection varies considerably according to the diagnostic technology used. Thus far, seroepidemiological data gained by the use of microimmunofluorescence, complement fixation tests or various EIAs have dominated the discussion. Schmidt et al. [30] reported that in Mecklenburg-Vorpommern, northeastern Germany, the prevalence of *Chlamydia pneumoniae* ARTI among children under 5 years of age was 9.6%, which is clearly higher than the 0.4% found in the present study. As reported by Verkooyen et al. [31] and Huniche et al. [32], *Chlamydia pneumoniae* cultures are readily contaminated by mycoplasma other than *Mycoplasma pneumoniae* (e.g. *Mycoplasma oralis*). Therefore, the prevalence of this organism may be exaggerated by serological data based on non-microimmunofluorescence tests.

The other possibility is that our technique may have underestimated the occurrence of *Chlamydia pneumoniae* infection. Normann et al. [33], for example, found the prevalence of PCR-positive, serologically confirmed *Chlamydia pneumoniae* infection in children under 5 years of age with ARTI to be 19% during an outbreak in Sweden that occurred between October 1994 and June 1996. These authors mentioned the superiority of throat swab specimens. The primer pair within the nucleotide sequence coding for the 16S ribosomal RNA that, according to Gaydos et al. [34], produces a 463 bp amplicon, was used in the multiplex RT-PCR. Testing of several laboratory samples of *Chlamydia pneumoniae* by single-primer PCR showed that the DNA preparation is not the limiting factor for sensitivity. The multiplex RT-PCR, however, has a 10- to 100-fold lower sensitivity in terms of concentration of pathogens within a given specimen (unpublished data). Jantos et al. [35] found three cases of *Chlamydia pneumoniae* infection among 290 infants and children with LRTI in Giessen, Germany, using PCR and serological tests. Increasing numbers of reports describe *Chlamydia pneumoniae* as a cause of URTI [33] or even as a colonizer of the upper respiratory tract [36, 37]. If upper respiratory tract carriage of *Chlamydia pneumoniae* is common, it could be difficult in the future to extrapolate upper respiratory tract findings to the lower respiratory tract, especially in epidemic situations. More recent reports, however, show a good correlation between PCR findings from URTI and serological findings from LRTI [33, 38].

In conclusion, the prevention of RSV infection should be of high priority. As 35% of the cases of RSV disease occurred in infants younger than 4 months of age, any vaccination approach and immunization strategy should take this important finding into consideration. When considering a combined RSV/PIV-3 vaccine, the very limited impact of PIV-3 infection in terms of absolute numbers must be taken into account. Since the distribution of pathogens within the different types of LRTI is similar, it seems that host factors determine which specific LRTI develops in an individual patient. Similar views were expressed earlier by Parrott et al. [39], who made the following statement at the Conference on Newer Respiratory Disease Viruses in 1963 at the National Institutes of Health: "Age-related anatomy, immunity and maternal antibodies and an individual predisposition seem to be involved".

Unlike cell culture techniques, multiplex RT-PCR does not require viable pathogens. It combines this advantage for epidemiological studies with speed for individual diagnosis. It also provides material for further analysis and storage. Therefore, multiplex RT-PCR is an appropriate epidemiological tool and might become the major technique for this type of analysis in the near future. It must be expanded to include additional pathogens and modified for *Chlamydia pneumoniae*.

In the long run, epidemiological research and surveillance, tailored to the regional level, should provide data to pediatricians and general practitioners to aid in the selection of appropriate antibiotic therapy.

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