Respiratory Pathogens in Children with and without Respiratory Symptoms

Marieke M. van der Zalm, MD, Bart E. van Ewijk, MD, PhD, Berry Wilbrink, PhD, Cuno S. P. M. Uiterwaal, MD, PhD, Tom F. W. Wolfs, MD, PhD, and Cornelis K. van der Ent, MD, PhD

Objectives To investigate the occurrence of respiratory pathogens in samples from children with and without respiratory symptoms and to identify whether age and/ or coinfections modify the impact of respiratory pathogens on symptoms.

Study design In a prospective longitudinal study, 18 children were sampled biweekly for respiratory pathogens, irrespective of respiratory symptoms. Polymerase chain reaction was performed for 13 respiratory pathogens. Episodes were defined "asymptomatic" if no symptoms of any respiratory tract illness were present between 1 week before and 1 week after sampling.

Results A total of 230 samples were collected. In 56% of the symptomatic episodes, a pathogen was detected, compared with 40% of the asymptomatic episodes ($P = .03$). Rhinovirus and coronaviruses were most prevalent in both symptomatic and asymptomatic episodes. In the youngest children, 9% of the pathogen-positive episodes were asymptomatic, compared with 36% in the oldest children ($P = .01$). Multiple pathogens were found in 17% of the symptomatic episodes and in 3% of the asymptomatic episodes ($P = .02$).

Conclusions Respiratory pathogens are frequently detected in samples from children with no respiratory symptoms. Symptomatic cases occurred more often in younger children and with detections of more than 1 respiratory pathogen. *(J Pediatr 2009;154:396-400)*

Respiratory tract infections occur frequently in early infancy and account for a major percentage of morbidity and mortality in childhood. Virus infections seem to be responsible for most of this burden. Since the introduction of molecular detection techniques, such as polymerase chain reaction (PCR), the percentage of pathogens found during respiratory tract illnesses in published studies has increased dramatically, up to 85%.[1-4]

Although many studies have investigated the prevalence of respiratory pathogens during respiratory illnesses, little is known about the prevalence of pathogens in nonsymptomatic children. Whether pathogens are actually the cause of the respiratory symptoms or are simply colonizing the respiratory tract during symptomatic episodes remains unclear. It can be speculated that not every infection with a pathogen leads to respiratory symptoms and that pathogenicity might depend on host or environmental factors. In young children, the respiratory and immune systems are immature and may be more susceptible to respiratory pathogens.[5] We hypothesized that infections with respiratory pathogens are likely to have the most serious effect on young children with developing respiratory and immunologic systems. Furthermore, we hypothesized that infection with multiple respiratory pathogens will more often lead to respiratory symptoms compared with infection with a single pathogen.[6-9]

The aims of our study were to determine the prevalence of respiratory pathogens in the presence or absence of respiratory tract symptoms in prospectively sampled young children, and to identify whether age and coinfections modify the impact of a pathogen on illness. Sensitive PCR techniques were used to detect 13 common respiratory pathogens, 11 viruses, and 2 atypical bacteria.

METHODS

Study Design and Subjects

A prospective longitudinal study was conducted during a 6-month winter season (November 2004 through April 2005). A total of 19 healthy children age 0 to 7 years were

PCR	Polymerase chain reaction	RSV	Respiratory syncytial virus

From the Divisions of Respiratory Diseases (M.vdZ., B.vE., C.vdE.) and Infectious Diseases (T.W.), Department of Pediatrics, Wilhelmina Children's Hospital, and Julius Center for Health Sciences and Primary Care (C.U.), University Medical Center Utrecht, Utrecht, The Netherlands and Laboratory of Infectious Diseases and Perinatal Screening, National Institute of Public Health and the Environment, Bilthoven, The Netherlands (B.W.).

Supported by a MD/PhD grant from the University Medical Center Utrecht, The Netherlands (to M.vdZ.) and a fellowship from the Wilhelmina Children's Hospital Research Fund (to B.vE.). None of the authors reports any conflict of interest.

Submitted for publication Feb 18, 2008; last revision received Jul 22, 2008; accepted Aug 18, 2008.

Reprint requests: Marieke M. van der Zalm, MD, Department of Pediatrics, Respiratory Diseases, Wilhelmina Children's Hospital, University Medical Centre Utrecht, PO Box 85090, Office KH 01.419.0, 3508 AB Utrecht, The Netherlands. E-mail: M.M.vanderzalm@umcutrecht.nl.

0022-3476/$ - see front matter

10.1016/j.jpeds.2008.08.036

enrolled, 1 of which failed to complete the study. None of the children had a history of asthma or recurrent respiratory complaints. Parents were contacted twice a week by telephone or e-mail by 1 of the 2 study coordinators to determine the presence of any symptoms of respiratory tract illness. Respiratory tract symptoms were defined as symptoms of coryza (rhinorrhea or nasal congestion), sore throat, earache with or without ear discharge, cough, sputum production, or dyspnea, all with or without temperature above 38°C.

Samples were collected every 2 weeks regardless of the presence or absence of respiratory symptoms. The biweekly sampling frequency during the study resulted in about 13 subsequent observation episodes per child. An episode was defined as "asymptomatic" if there were no respiratory symptoms during a complete period of 1 week before to 1 week after sampling. An episode was defined as "symptomatic" if there were any respiratory symptoms during the period of 1 week before to 1 week after sampling.

The study design was approved by the local Medical Ethics Committee, and all parents gave written informed consent.

Detection of Respiratory Pathogens

Respiratory pathogens were detected by PCR. After receiving precise instruction at the beginning of the study, parents collected the samples by rubbing 1 of the child's nostrils and the posterior oropharynx using separate cotton-tipped swabs. After sampling, the 2 swabs were collected into a single vial containing GLY medium with 0.1 mg/mL of pimaricine as a viral transport medium and sent to our laboratory by regular mail. Samples were stored at −20°C until analysis. Sampling of respiratory pathogens by parents using nose and throat swabs has proven feasible and reliable. Both the sampling frequency and the viral recovery rate in parental samples are higher compared with sampling by a dedicated research nurse.[10,11]

PCR was performed at the National Institute of Public Health and the Environment, Bilthoven, The Netherlands. The respiratory pathogens human rhinovirus and enterovirus, human metapneumovirus, human coronaviruses OC43 and 229E, *Chlamydophila pneumoniae*, and *Mycoplasma pneumoniae* were analyzed by conventional PCR, essentially as described previously.[3]

The PCR for adenovirus consisted of 40 cycles of 1 minute at 94°C, 1.5 minute at 45°C, 1 minute at 72°C with a final extension of 10 minutes at 72°C (PE 9700) with the following primers: forward, 5′-GCCGCAGTGGTCTTACATGCACAT-3′; reverse, 5′-ARCACICCICGRATGTCAAAG-3′ and 5′-CAGCACGCCGCGGATGTCAAAGT-3′ targeting the hexon gene. Amplicons were analyzed by gel electrophoresis.

Real-time PCR for human coronavirus NL63, influenza viruses A and B, and respiratory syncytial virus (RSV) A and B was performed using the Lightcycler 2.0 format with Lightcycler Taqman Mastermix (Roche, Germany). A separate reverse-transcription step with avian myeloblastosis virus reverse transcriptase was used for 60 min at 42°C for NL63 and for 60 minutes at 50°C for influenza and RSV.

The reaction for NL63 consisted of 1 cycle of 10 minutes at 95°C and 45 cycles of 5 seconds at 50°C and 10 seconds at 72°C, with primers 5′-AACCTAATAAGCCTCTTTCTC-3′ and 5′-TTTGGCATCACCATTCTG-3′ and probe 5′-6FAM-AGTGCTTTGGTCCTCGTG-Tamra-3′ targeting the nucleocapsid gene, as provided by L. van der Hoek.[12]

The reaction for influenza consisted of 1 cycle of 10 minutes at 95°C, and 45 cycles of 10 seconds at 95°C, 20 seconds at 50°C, and 10 seconds at 72°C, with primers 5′-AAGACCAATCCTGTCACCTCTGA-3′ and 5′-CAAAGCGTCTACGCTGCAGTCC-3′ with probe 5′-6Fam-TTTGTGTTCACGCTCACCGTGCC-BHQ1-3′ for influenza A, targeting the *M pneumoniae* gene, and 5′-TGAAGGACATTCAAAGC-3′ and 5′-ACCAGTCTAATTGTCTC-3′ with probe 5′-YY-AGCACCGATTACACCAG-BHQ1-3′ for influenza B, targeting the NS gene.

The reaction for RSV consisted of I cycle of 10 minutes at 95°C and 45 cycles of 15 seconds at 95°C and 47 seconds at 60°C with primers 5′-TGAACAACCCAAAAGCATCA-3′ and 5′-CCTAGGCCAGCA GCATTG-3′ with probe 5′-6Fam-AATTTCCTCACTTCTCCAGTGTAGTATTAGG-BHQ1-3′ for RSV A and 5′-TGTCAATATTATCTCCTGTACTACGTTGAA-3′ and 5-GATGGCTCTTAGCAAAGTCAAGTTAA-3′ with probe 5′-YY-TGATACATTAAATAAGGATCAGCTGCTGTCATCCA-BHQ1-3′ for RSV B, both targeting the nucleocapsid gene.

Each sample was spiked with the equine arteritis virus as an internal control to detect inhibition of RNA isolation and PCR.[13]

Table I. Characteristics of the study group (n = 18)

Sex, M/F	3/15
Age, years	3.67 (0-7)
0-2	8
3-4	6
5-7	4
Number of siblings/child	1.50 (0-4)
Number of symptomatic episodes/child	9.50 (4-15)
Number of asymptomatic infections/child	3.00 (0-9)

Data are expressed as median and range in parentheses.

Statistical Analysis

All statistical analyses were done using SPSS version 12.0 (SPSS Inc, Chicago, Illinois). A 2-tailed χ^2 test was used to compare differences between groups. Differences were considered statistically significant at a P value $\leq .05$.

RESULTS

During a period of 460 child-weeks, respiratory symptoms in were recorded in 18 children. There were 2 sibling