Molecular detection of respiratory viruses: clinical impact

Colofon:
Frits Castelijns (Castelijns Grafische Projekten) en Camiel Lintsen (Kade05) are kindly acknowledged for the lay-out of this thesis.

Production: Helms Studio BV, Eindhoven

Printing: Gildeprint BV, Enschede

ISBN: 978-90-393-5224-3

Printing of this thesis was financially supported by: Ballast Nedam Bouw Speciale Projecten, Boehringen Ingelheim, Grünenthal B.V., Infection and Immunity Center Utrecht, J.E. Jurriaanse Stichting, Mead Johnson, Mevrouw T. de Vries-Kruyt, NYCOMED BV, the Netherlands Society of Medical Microbiology (NVMM) and the Netherlands Society for Microbiology (NVvM), and the University Medical Center Utrecht.

Molecular detection of respiratory viruses: clinical impact

Moleculaire detectie van respiratoire virussen: klinische implicaties

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op woensdag 9 december 2009 des middags te 2.30 uur

door

Alma Cécile van de Pol

geboren op 16 maart 1980, te Eindhoven

Promotor	Prof.dr. J.L.L. Kimpen
Co-promotoren	Dr. N.J.G. Jansen Dr. J.W.A. Rossen Dr. T.F.W. Wolfs

Contents

CHAPTER 1

General introduction

Lower respiratory tract infections in infancy

Lower respiratory tract infections (LRTIs) in infants and young children are a major public health problem. Worldwide, they cause more disease and deaths than any other infection (1;2). Every year, 2% of infants is admitted to the hospital with LRTI (3;4). In the group of infants that are hospitalized with LRTI, 2% becomes severely ill, with respiratory failure requiring mechanical ventilation at the pediatric intensive care unit (PICU) (5). LRTI is the most common cause for admission to the PICU, especially during the winter season (6).

LRTI in children can be divided into two clinical syndromes: bronchiolitis and pneumonia. Differentiation of these two entities in young children is difficult because standardized clinical criteria are not available (7). Bronchiolitis is an infection of the small airways in infants and young children, characterized by coryza, cough, tachypnea, retractions, wheezing and hyperinflation (4). Pneumonia is an infection of lung parenchyma, and is common both in children and adults. Patients with pneumonia usually present with fever, acute respiratory symptoms, and a rise of infection parameters in the blood (1). On chest radiograph, pneumonia is associated with focal infiltration, whereas bronchiolitis is associated with hyperinfiltration, atelectasis and patchy infiltration, distributed diffusely over both lungs (7). Since features of bronchiolitis and pneumonia overlap in infants and young children, the term LRTI is used in this thesis to denote either one of the two entities.

Historically, LRTIs is often differentiated into infections of viral and bacterial origin, with bronchiolitis being associated with a viral, and pneumonia with a bacterial cause. However, on clinical ground alone, a distinction between viral and bacterial LRTI cannot be made. In immunocompetent infants and children, it is generally believed that the majority of LRTIs are of viral origin. Viral tests such as viral culture, direct immunofluorescence (DIF), and lately real-time PCR are often being used to make an etiological viral diagnosis (8).

Viral detection methods

Viral culture

Viral culture was discovered in the beginning of the twentieth century, when it became possible to propagate single cells for viral inoculation. By the 1970s commercially prepared cell lines for respiratory viruses became available (9). In culture, viral growth can be detected microscopically

as degenerative changes in cell monolayers (i.e. cytopathogenic effect of the virus; Figure 1). Cell culture is an inexpensive technique, and it detects infectious viral particles only. However, viral culture is labor intensive, and not all respiratory viruses grow well in vitro. Furthermore, viral culture has limited sensitivity, and specimens for viral culture are highly susceptible to adverse storage and transportation conditions. Finally, viral culture has a long turn-around time (1-2 weeks).

Direct immunofluorescence

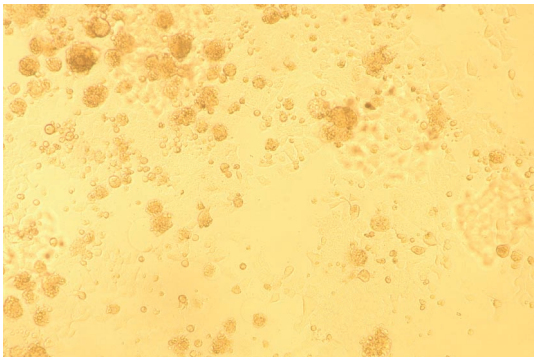
For clinical purposes, rapid viral tests are needed. DIF was one of the first methods permitting rapid viral diagnosis (1970s) (10). In DIF assays fluorescence-labeled antibodies are used to bind viral antigens in a clinical specimen. Fluorescent staining of bound antigens can be seen under a microscope with ultra-violet illumination (Figure 2). Advantages of DIF are low costs and high speed (turn-around time <1 day). However, similar to viral culture, DIF has drawbacks such as labor intensiveness, availability only for a limited number of viruses, and low sensitivity.

Molecular detection

In the 1980s the diagnostic virology field was revolutionized with the discovery that nucleic acids (i.e. DNA or RNA) could be amplified in a chain reaction using polymerases (11). With molecular methods such as polymerase chain reaction (PCR) a large number of identical DNA copies can be obtained for analysis. The PCR amplification consists of multiple three-step cycles: (1) double-stranded (c)DNA is separated (denaturation); (2) two different primers anneal to the complementary (c)DNA templates (annealing); and (3) polymerase elongates the primer, synthesizing new DNA strands (elongation). Conventionally, after a predefined number of cycles, amplified DNA is visualized using gel electrophoresis. Real-time PCR, a variant of PCR in which the quantity of amplified DNA is monitored during the course of the PCR reaction, became available in the 1990s. In real-time PCR reactions fluorescently labeled probes are used that emit light signals proportional to the quantity of amplified DNA (Figure 3). Advantages of real-time PCR are numerous, including high analytical sensitivity, short turn-around time (1-2 days), detection of amplified DNA after every cycle, the generation of semi-quantitative results (12-15), and availability for all known respiratory viruses. As real-time PCR uses closed-tube detection systems, the risk of carry-over contamination is minimized (i.e. false positive results are highly unlikely). A disadvantage of PCR as diagnostic method for viruses is the high costs of this method. In addition, another possible disadvantage may be the fact that it detects not only infectious virus but also non-infectious viruses and nucleic acid remains.
Molecular methods such as PCR made it possible to discover new viruses that cannot replicate in

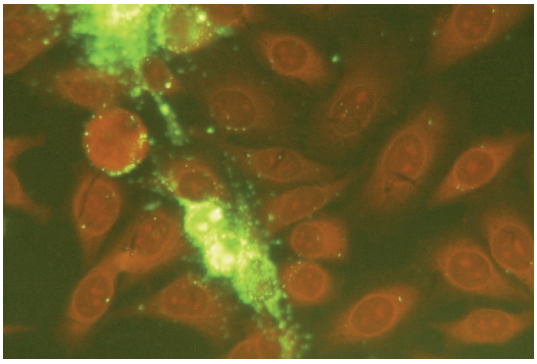
vitro. With random PCR all nucleic acids present in a specimen can be amplified, and amplified DNA can subsequently be sequenced. Obtained sequences can be compared with bioinformatics to previously known sequences in databases. This way, a differentiation can be made between known sequences and unknown sequences, the latter possibly belonging to thus far unknown new viruses. Using molecular methods, several new viruses have been discovered since 2005.

Figure 1 Viral culture: CPE in a respiratory specimen from a young child with RSV-LRTI



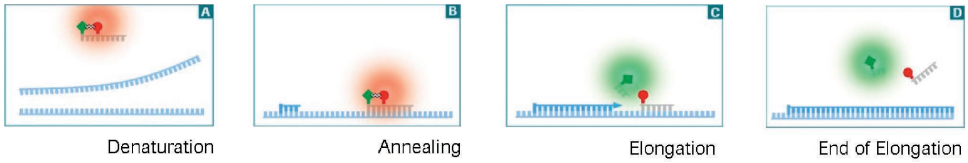
Typical CPE of RSV is seen in a Hep-2 cell monolayer through a microscope (CPE is most clearly visible as the "pancake like" structures in the left top of the image). Abbreviations: CPE: cytopathogenic effect; LRTI: lower respiratory tract infection. The picture was kindly provided by Ernst-Jan Jacobsen from the Department of Virology, University Medical Center Utrecht.

Figure 2 Direct immunofluorescence: fluorescent staining in a respiratory specimen from a young child with RSV-LRTI



Fluorescent staining of RSV antigens is seen in epithelial cells through a microscope (fluorescence is visible as bright green dots). Abbreviations: LRTI: lower respiratory tract infection. The picture was kindly provided by Ernst-Jan Jacobsen from the Department of Virology, University Medical Center Utrecht.

Figure 3 Real-time PCR



A: Double-stranded (c)DNA is separated (denaturation). A fluorescently labeled probe is used with a quencher (red) suppressing the reporter fluorescence signal (green).
B: primers and probes specifically anneal to the target sequence (annealing).
C: As polymerase elongates the primer, synthesizing new DNA strands (elongation), it encounters the probe. Polymerase than cleaves the probe, displaces the probe fragments from the target, and continues to synthesize new DNA.
D: In the cleaved probe, the reporter dye is no longer suppressed by the quencher and emits light. The amount of light is proportional to the quantity of amplified DNA. The graph was kindly provided by Roche Diagnostics.

Epidemiology of respiratory viruses

Historically, respiratory syncytial virus A and B (RSV), influenza virus A and B (IV), parainfluenza virus 1 to 4 (PIV), adenovirus (AdV), human rhinovirus (hRV), and human coronavirus 229E and OC43 (hCoV) are regarded the most common respiratory viruses in children with LRTI. The latter two viruses can only be detected easily with real-time PCR.

RSV is generally accepted as the predominant cause of bronchiolitis in young children (3). Virtually all children have developed antibodies to RSV by the age of 2 years (7;16). RSV is a seasonal virus, with peak rates of infection occurring annually in the winter season in temperate climates. Risk factors for severe RSV LRTI include young age, prematurity, chronic lung disease, congenital heart disease, Down syndrome, and immunodeficiency (17;18). Like RSV, IV, PIV, and AdV are also generally accepted as common pathogens in LRTI (19-23). Patients hospitalized with IV, PIV, and AdV are on average somewhat older than RSV patients (24-26). IV activity peaks in the winter season, whereas PIV is more prevalent during fall and spring (25).

HRV was long believed to be a causative agent of upper, and not lower, respiratory tract infection. However, with the use of molecular methods this concept has changed, and numerous studies have now shown that hRV is the most frequent virus in hospitalized LRTI patients under

five years of age (27-29). HRV has also gained interest as the major cause of asthma exacerbations. Asthmatic children seem to be predisposed to more severe disease upon infection with hRV, compared to non-asthmatic controls (30;31).

Over the past 8 years several new viruses have been added to the list of respiratory viruses. In 2001 human metapneumovirus (hMPV) was discovered, followed by the new hCoV NL63 in 2004 (32;33). Both hMPV and hCoV NL63 are now recognized to have global presence, and are generally considered causative agents of LRTI (34). From 2005 to 2008 three new viruses have been discovered: human bocavirus (hBoV), KI polyomavirus (KIPyV) and WU polyomavirus (WUPyV) (35-37). Whether these three new viruses are causally associated with LRTI in infants and young children remains to be determined. All new respiratory viruses can only be detected routinely with real-time PCR, since they do not grow well in culture and there are no commercial DIF assays available for their identification.

Several studies have investigated the epidemiology of the different respiratory viruses, and depending on the setting (i.e. outpatient or inpatient setting), age, and type of viral test the prevalence of RSV, IV, PIV, AdV, hRV, hCoV, hMPV, hBoV, KIPyV, and WUPyV in patients with respiratory infections is estimated to be approximately 40%, 5%, 10%, 10%, 40%, 5-10%, 5-10%, 10%, 0-5%, 0-5%, respectively (22;25;34;38-40). With the use of molecular methods, the prevalence of multiple viral infection is around 20-35% (27;40-43).

Clinical implications of molecular methods

Etiology

With the introduction of highly sensitive molecular methods such as real-time PCR assays for a wide range of respiratory viruses the yield of viruses in patients with LRTI is greatly increased. However, it is not clear whether all detected viruses truly represent etiological pathogens in each patient with LRTI. Nucleic acids of viruses found by real-time PCR could theoretically represent asymptomatic carriership, non-replicating viruses, or viral remnants, especially if the nucleic acids are found in minute quantities. Research on the etiologic role of microbes has its origin in 1880, when Robert Koch formulated his postulates to determine that a micro organism is the cause of disease: (1) the microbe is present in every case of the disease; (2) the microbe is present in no other disease; (3) after being isolated in culture, it can induce the disease anew. For many viruses these postulates could never be fulfilled because of poor replication in viral culture. In an era of rapidly evolving molecular technology, new guidelines are needed to

establish causation (34). Insight in the causal role of viruses in LRTI patients requires epidemiologic data on virus prevalence, and insight in the temporal relations of exposure and symptoms (44).

Patient management at the PICU

Currently, molecular methods are being implemented in clinical laboratories worldwide (12;14;45). At the PICU, real-time PCR for respiratory viruses is increasingly used in the routine diagnostic work-up of immunocompetent infants and children with LRTI (28;46;47;47;48). However, whether real-time PCR influences clinical decision making in this patient group remains to be determined. On clinical grounds, the distinction between viral and bacterial LRTI cannot be made, and previous studies hypothesized that rapid detection of respiratory viruses could provide clinicians with a sufficient explanation for respiratory symptoms, and antibiotics might not be prescribed. However, the results from these studies, all performed at the emergency department or the general ward, were controversial (49-54). Evidence on the impact of real-time PCR testing of LRTI patients at the PICU is needed, because if PCR does not affect daily practice in this setting, it is not useful to use it routinely, and costs could be saved.

Pathofysiology

It is currently unclear why a minority of infants and young children becomes severely ill upon infection with respiratory viruses, whereas most children experience only mild symptoms. Disease severity may be determined by viral factors or by the nature of the immune response. In RSV disease, viral factors and immune determinants have extensively been studied. However, it is still debated whether the enhanced disease in RSV patients at the PICU is caused by viral factors or the immune response (55-57). Since the 1960s it has been suggested that RSV is merely an immune mediated disease. Early mouse models showed that CD8+ T cell depletion results in reduction of disease (58;59). Conversely, other observations indicate a viral-induced pathogenesis of RSV disease. Severe RSV LRTI is more frequent in immune compromised patients (55;60), and maternal antibodies ameliorate rather than enhance disease (55). Furthermore, lung tissue from infants with fatal RSV LRTI has been shown to harbor numerous viral particles in absence of CD8+ T lymphocytes (61). As human pathogenesis studies are unethical, and animal studies are poor surrogates, the controversy of viral and immune contribution needs to be addressed during the natural disease course of RSV (12;62;63). PICU patients are a unique study group in this context, since both the lower airways and the circulation are relatively easily accessible. Reliable quantitative RSV detection may be paramount to elucidate the kinetics of symptom severity, viral load, and immune response in PICU patients with RSV LRTI.

Aims of the thesis

- To investigate the epidemiology of respiratory viruses in PICU patients with LRTI using conventional tests (viral culture and DIF) and real-time PCR.
- To evaluate whether the implementation of real-time PCR, additional to DIF, in the work-up of PICU patients with LRTI impacts the use of antibiotics.
- To determine whether quantitative real-time PCR in respiratory samples can be used to gain insight in the pathogenic role of RSV in LRTI.

Outline of the thesis

Detection of respiratory viruses

In *chapter 2* the sensitivity and specificity of conventional respiratory viral tests (viral culture and DIF) for RSV, IV, PIV, and AdV is calculated, as compared with real-time PCR as the reference method, in samples from hospitalized children and adults with respiratory symptoms. In *chapter 3* the diagnostic yield of conventional tests and real-time PCR was evaluated in children and infants with LRTI at the PICU. To study the prevalence of a broad range of respiratory viruses real-time PCR was not only used to detect RSV, IV, PIV, and AdV, but also to detect hRV, CoV 229E, OC43, and NL63, and hMPV. In *chapter 4* the international literature is systematically reviewed to determine the sensitivity and specificity of conventional respiratory viral tests compared to PCR as the reference method. The prevalence of the newly detected viruses hBoV, KIPyV, and WUPyV at the PICU, in LRTI patients and controls, is assessed in *chapter 5*.

Impact on patient management

The impact of respiratory viral real-time PCR on antibiotic use of LRTI patients at the PICU is addressed in a paper case study and a prospective study described in *chapter 6*. In *chapter 7* real-time PCR is used to prospectively study the frequency of RSV transmission at the PICU.

Pathofysiology of RSV

Chapter 8 evaluates the use of real-time PCR for quantitative detection of RSV RNA. The variation of RSV RNA load in one single sample, the variation in two samples during one day, and the variation between the upper and the lower airways is reported. Finally, in *chapter 9*, quantitative RSV real-time PCR is used to study the kinetics of disease severity, viral load, and innate and adaptive immune activation in infants with RSV LRTI at the PICU.
In *chapter 10* the results of the studies are discussed. *Chapter 11* provides an English and a Dutch summary.

Reference List

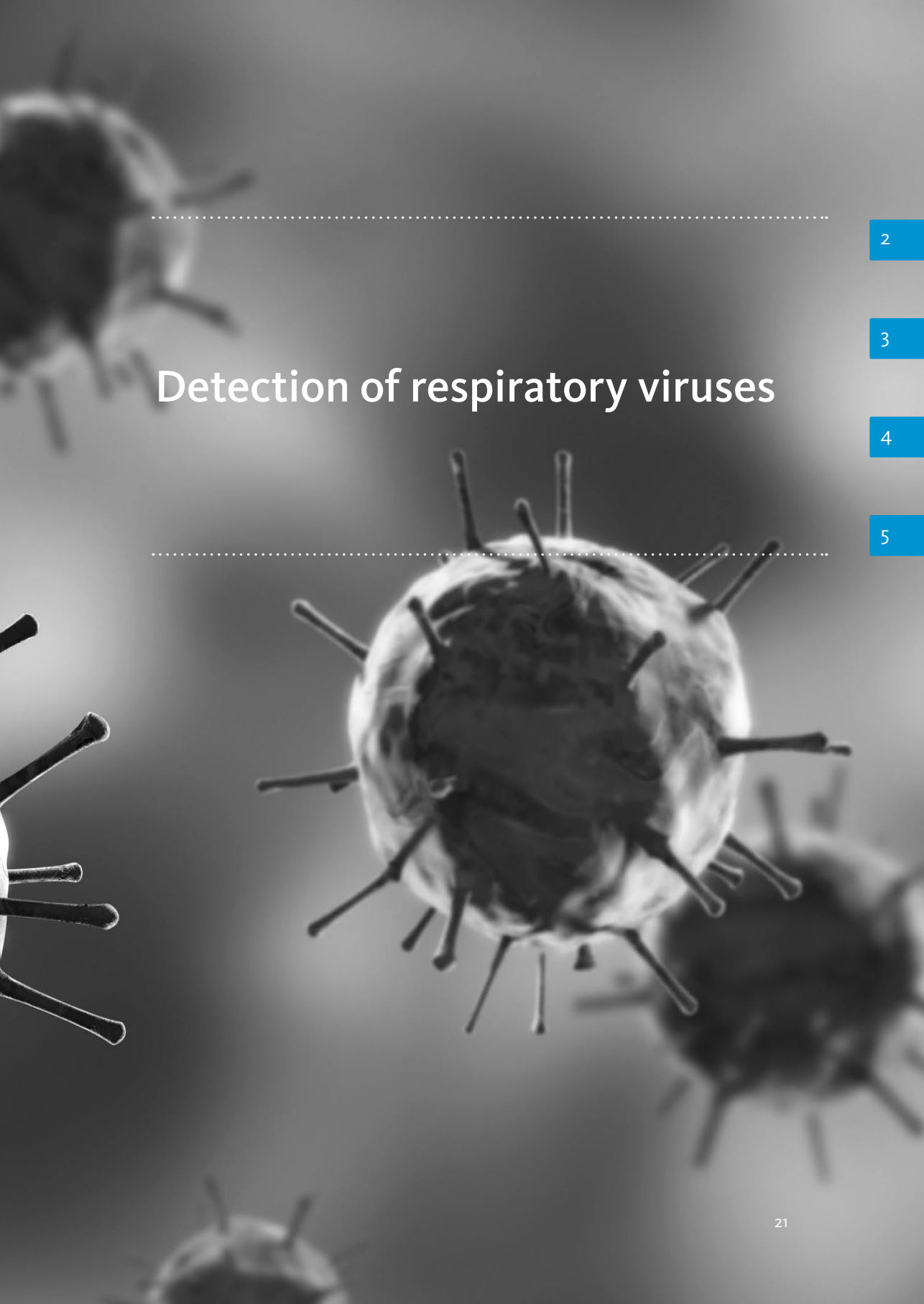
(1) McIntosh K. Community-acquired pneumonia in children. N Engl J Med 2002 February 7;346(6):429-37.
(2) Mizgerd JP. Lung infection--a public health priority. PLoS Med 2006 February;3(2):e76.
(3) Hall CB, Weinberg GA, Iwane MK, Blumkin AK, Edwards KM, Staat MA, Auinger P, Griffin MR, Poehling KA, Erdman D, Grijalva CG, Zhu Y, Szilagyi P. The burden of respiratory syncytial virus infection in young children. N Engl J Med 2009 February 5;360(6):588-98.
(4) Smyth RL, Openshaw PJ. Bronchiolitis. Lancet 2006 July 22;368(9532):312-22.
(5) Shay DK, Holman RC, Newman RD, Liu LL, Stout JW, Anderson LJ. Bronchiolitis-associated hospitalizations among US children, 1980-1996. JAMA 1999 October 20;282(15):1440-6.
(6) Randolph AG, Meert KL, O'Neil ME, Hanson JH, Luckett PM, Arnold JH, Gedeit RG, Cox PN, Roberts JS, Venkataraman ST, Forbes PW, Cheifetz IM. The feasibility of conducting clinical trials in infants and children with acute respiratory failure. Am J Respir Crit Care Med 2003 May 15;167(10):1334-40.
(7) van Woensel JB, van Aalderen WM, Kimpen JL. Viral lower respiratory tract infection in infants and young children. BMJ 2003 July 5;327(7405):36-40.
(8) Bordley WC, Viswanathan M, King VJ, Sutton SF, Jackman AM, Sterling L, Lohr KN. Diagnosis and testing in bronchiolitis: a systematic review. Arch Pediatr Adolesc Med 2004 February;158(2):119-26.
(9) Leland DS, Ginocchio CC. Role of cell culture for virus detection in the age of technology. Clin Microbiol Rev 2007 January;20(1):49-78.
(10) Madeley CR, Peiris JS. Methods in virus diagnosis: immunofluorescence revisited. J Clin Virol 2002 August;25(2):121-34.
(11) Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 1985 December 20;230(4732):1350-4.

(12) Ieven M. Currently used nucleic acid amplification tests for the detection of viruses and atypicals in acute respiratory infections. J Clin Virol 2007 December;40(4):259-76.
(13) Kubista M, Andrade JM, Bengtsson M, Forootan A, Jonak J, Lind K, Sindelka R, Sjoback R, Sjogreen B, Strombom L, Stahlberg A, Zoric N. The real-time polymerase chain reaction. Mol Aspects Med 2006 April;27(2-3):95-125.
(14) Mackay IM. Real-time PCR in the microbiology laboratory. Clin Microbiol Infect 2004 March;10(3):190-212.
(15) Niesters HG. Clinical virology in real time. J Clin Virol 2002 December;25 Suppl 3:S3-12.
(16) Simoes EA. Respiratory syncytial virus infection. Lancet 1999 September 4;354(9181):847-52.
(17) Bloemers BL, van Furth AM, Weijerman ME, Gemke RJ, Broers CJ, van den EK, Kimpen JL, Strengers JL, Bont LJ. Down syndrome: a novel risk factor for respiratory syncytial virus bronchiolitis--a prospective birth-cohort study.
Pediatrics 2007 October;120(4):e1076-e1081.
(18) Faber TE, Kimpen JL, Bont LJ. Respiratory syncytial virus bronchiolitis: prevention and treatment. Expert Opin Pharmacother 2008 October;9(14):2451-8.
(19) Poehling KA, Edwards KM, Weinberg GA, Szilagyi P, Staat MA, Iwane MK, Bridges CB, Grijalva CG, Zhu Y, Bernstein DI, Herrera G, Erdman D, Hall CB, Seither R, Griffin MR. The underrecognized burden of influenza in young children. N Engl J Med 2006 July 6;355(1):31-40.
(20) Weinberg GA, Hall CB, Iwane MK, Poehling KA, Edwards KM, Griffin MR, Staat MA, Curns AT, Erdman DD, Szilagyi PG. Parainfluenza virus infection of young children: estimates of the population-based burden of hospitalization. J Pediatr 2009 May;154(5):694-9.
(21) Gardner PS. Virus infections and respiratory disease of childhood. Arch Dis Child 1968 December;43(232):629-45.
(22) Jartti T, Lehtinen P, Vuorinen T, Osterback R, van den HB, Osterhaus AD, Ruuskanen O. Respiratory picornaviruses and respiratory syncytial virus as causative agents of acute expiratory wheezing in children. Emerg Infect Dis 2004 June;10(6):1095-101.
(23) Juven T, Mertsola J, Waris M, Leinonen M, Meurman O, Roivainen M, Eskola J, Saikku P, Ruuskanen O. Etiology of community-acquired pneumonia in 254 hospitalized children. Pediatr Infect Dis J 2000 April;19(4):293-8.
(24) Bosis S, Esposito S, Niesters HG, Crovari P, Osterhaus AD, Principi N. Impact of human metapneumovirus in childhood: comparison with respiratory syncytial virus and influenza viruses. J Med Virol 2005 January;75(1):101-4.
(25) Iwane MK, Edwards KM, Szilagyi PG, Walker FJ, Griffin MR, Weinberg GA, Coulen C, Poehling KA, Shone LP, Balter S, Hall CB, Erdman DD, Wooten K, Schwartz B. Population-based surveillance for hospitalizations associated with respiratory syncytial virus, influenza virus, and parainfluenza viruses among young children. Pediatrics 2004 June;113(6):1758-64.

(26) Meury S, Zeller S, Heininger U. Comparison of clinical characteristics of influenza and respiratory syncytial virus infection in hospitalised children and adolescents. Eur J Pediatr 2004 July;163(7):359-63.
(27) Jartti T, Lehtinen P, Vuorinen T, Ruuskanen O. Bronchiolitis: age and previous wheezing episodes are linked to viral etiology and atopic characteristics. Pediatr Infect Dis J 2009 April;28(4):311-7.
(28) Louie JK, Roy-Burman A, Guardia-Labar L, Boston EJ, Kiang D, Padilla T, Yagi S, Messenger S, Petru AM, Glaser CA, Schnurr DP. Rhinovirus associated with severe lower respiratory tract infections in children. Pediatr Infect Dis J 2009 April;28(4):337-9.
(29) Miller EK, Lu X, Erdman DD, Poehling KA, Zhu Y, Griffin MR, Hartert TV, Anderson LJ, Weinberg GA, Hall CB, Iwane MK, Edwards KM. Rhinovirus-associated hospitalizations in young children. J Infect Dis 2007 March 15;195(6):773-81.
(30) Corne JM, Marshall C, Smith S, Schreiber J, Sanderson G, Holgate ST, Johnston SL. Frequency, severity, and duration of rhinovirus infections in asthmatic and non-asthmatic individuals: a longitudinal cohort study. Lancet 2002 March 9;359(9309):831-4.
(31) Heymann PW, Platts-Mills TA, Johnston SL. Role of viral infections, atopy and antiviral immunity in the etiology of wheezing exacerbations among children and young adults. Pediatr Infect Dis J 2005 November;24(11 Suppl):S217-22, discussion.
(32) van den Hoogen BG, de Jong JC, Groen J, Kuiken T, de GR, Fouchier RA, Osterhaus AD. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. Nat Med 2001 June;7(6):719-24.
(33) van der Hoek L, Pyrc K, Jebbink MF, Vermeulen-Oost W, Berkhout RJ, Wolthers KC, Wertheim-van Dillen PM, Kaandorp J, Spaargaren J, Berkhout B. Identification of a new human coronavirus. Nat Med 2004 April;10(4):368-73.
(34) Sloots TP, Whiley DM, Lambert SB, Nissen MD. Emerging respiratory agents: new viruses for old diseases? J Clin Virol 2008 July;42(3):233-43.
(35) Allander T, Tammi MT, Eriksson M, Bjerkner A, Tiveljung-Lindell A, Andersson B. Cloning of a human parvovirus by molecular screening of respiratory tract samples. Proc Natl Acad Sci U S A 2005 September 6;102(36):12891-6.
(36) Allander T, Andreasson K, Gupta S, Bjerkner A, Bogdanovic G, Persson MA, Dalianis T, Ramqvist T, Andersson B. Identification of a third human polyomavirus. J Virol 2007 April;81(8):4130-6.
(37) Gaynor AM, Nissen MD, Whiley DM, Mackay IM, Lambert SB, Wu G, Brennan DC, Storch GA, Sloots TP, Wang D. Identification of a novel polyomavirus from patients with acute respiratory tract infections. PLoS Pathog 2007 May 4;3(5):e64.
(38) Allander T, Jartti T, Gupta S, Niesters HG, Lehtinen P, Osterback R, Vuorinen T, Waris M, Bjerkner A, Tiveljung-Lindell A, van den Hoogen BG, Hyypia T, Ruuskanen O. Human bocavirus and acute wheezing in children. Clin Infect Dis 2007 April 1;44(7):904-10.

(39) Arnold JC, Singh KK, Spector SA, Sawyer MH. Undiagnosed respiratory viruses in children. Pediatrics 2008 March;121(3):e631-e637.
(40) Jennings LC, Anderson TP, Werno AM, Beynon KA, Murdoch DR. Viral etiology of acute respiratory tract infections in children presenting to hospital: role of polymerase chain reaction and demonstration of multiple infections. Pediatr Infect Dis J 2004 November;23(11):1003-7.
(41) Aberle JH, Aberle SW, Pracher E, Hutter HP, Kundi M, Popow-Kraupp T. Single versus dual respiratory virus infections in hospitalized infants: impact on clinical course of disease and interferon-gamma response. Pediatr Infect Dis J 2005 July;24(7):605-10.
(42) Drews AL, Atmar RL, Glezen WP, Baxter BD, Piedra PA, Greenberg SB. Dual respiratory virus infections. Clin Infect Dis 1997 December;25(6):1421-9.
(43) Paranhos-Baccala G, Komurian-Pradel F, Richard N, Vernet G, Lina B, Floret D. Mixed respiratory virus infections. J Clin Virol 2008 December;43(4):407-10.
(44) Fredericks DN, Relman DA. Sequence-based identification of microbial pathogens: a reconsideration of Koch's postulates. Clin Microbiol Rev 1996 January;9(1):18-33.
(45) Espy MJ, Uhl JR, Sloan LM, Buckwalter SP, Jones MF, Vetter EA, Yao JD, Wengenack NL, Rosenblatt JE, Cockerill FR, III, Smith TF. Real-time PCR in clinical microbiology: applications for routine laboratory testing. Clin Microbiol Rev 2006 January;19(1):165-256.
(46) Harper SA, Bradley JS, Englund JA, File TM, Gravenstein S, Hayden FG, McGeer AJ, Neuzil KM, Pavia AT, Tapper ML, Uyeki TM, Zimmerman RK. Seasonal influenza in adults and children--diagnosis, treatment, chemoprophylaxis, and institutional outbreak management: clinical practice guidelines of the Infectious Diseases Society of America. Clin Infect Dis 2009 April 15;48(8):1003-32.
(47) Crowcroft NS, Zambon M, Harrison TG, Mok Q, Heath P, Miller E. Respiratory syncytial virus infection in infants admitted to paediatric intensive care units in London, and in their families. Eur J Pediatr 2008 April;167(4):395-9.
(48) Gagneur A, Vallet S, Talbot PJ, Legrand-Quillien MC, Picard B, Payan C, Sizun J. Outbreaks of human coronavirus in a pediatric and neonatal intensive care unit. Eur J Pediatr 2008 December;167(12):1427-34.
(49) Bonner AB, Monroe KW, Talley LI, Klasner AE, Kimberlin DW. Impact of the rapid diagnosis of influenza on physician decision-making and patient management in the pediatric emergency department: results of a randomized, prospective, controlled trial. Pediatrics 2003 August;112(2):363-7.
(50) Doan QH, Kissoon N, Dobson S, Whitehouse S, Cochrane D, Schmidt B, Thomas E. A randomized, controlled trial of the impact of early and rapid diagnosis of viral infections in children brought to an emergency department with febrile respiratory tract illnesses. J Pediatr 2009 January;154(1):91-5.
(51) Noyola DE, Demmler GJ. Effect of rapid diagnosis on management of influenza A infections. Pediatr Infect Dis J 2000 April;19(4):303-7.

(52) Poehling KA, Zhu Y, Tang YW, Edwards K. Accuracy and impact of a point-of-care rapid influenza test in young children with respiratory illnesses. Arch Pediatr Adolesc Med 2006 July;160(7):713-8.
(53) Sharma V, Dowd MD, Slaughter AJ, Simon SD. Effect of rapid diagnosis of influenza virus type a on the emergency department management of febrile infants and toddlers. Arch Pediatr Adolesc Med 2002 January;156(1):41-3.
(54) Woo PC, Chiu SS, Seto WH, Peiris M. Cost-effectiveness of rapid diagnosis of viral respiratory tract infections in pediatric patients. J Clin Microbiol 1997 June;35(6):1579-81.
(55) Collins PL, Graham BS. Viral and host factors in human respiratory syncytial virus pathogenesis. J Virol 2008 March;82(5):2040-55.
(56) Openshaw PJ. Antiviral immune responses and lung inflammation after respiratory syncytial virus infection. Proc Am Thorac Soc 2005;2(2):121-5.
(57) Verbsky JW, Grossman WJ. RSV infection--an immune balancing act: commentary on the article by Bem et al. on page 650. Pediatr Res 2008 June;63(6):599-601.
(58) Cannon MJ, Openshaw PJ, Askonas BA. Cytotoxic T cells clear virus but augment lung pathology in mice infected with respiratory syncytial virus. J Exp Med 1988 September 1;168(3):1163-8.
(59) Graham BS, Bunton LA, Wright PF, Karzon DT. Role of T lymphocyte subsets in the pathogenesis of primary infection and rechallenge with respiratory syncytial virus in mice. J Clin Invest 1991 September;88(3):1026-33.
(60) Welliver RC. Review of epidemiology and clinical risk factors for severe respiratory syncytial virus (RSV) infection. J Pediatr 2003 November;143(5 Suppl):S112-S117.
(61) Welliver TP, Garofalo RP, Hosakote Y, Hintz KH, Avendano L, Sanchez K, Velozo L, Jafri H, Chavez-Bueno S, Ogra PL, McKinney L, Reed JL, Welliver RC, Sr. Severe human lower respiratory tract illness caused by respiratory syncytial virus and influenza virus is characterized by the absence of pulmonary cytotoxic lymphocyte responses. J Infect Dis 2007 April 15;195(8):1126-36.
(62) DeVincenzo JP, El Saleeby CM, Bush AJ. Respiratory syncytial virus load predicts disease severity in previously healthy infants. J Infect Dis 2005 June 1;191(11):1861-8.
(63) Wright PF, Gruber WC, Peters M, Reed G, Zhu Y, Robinson F, Coleman-Dockery S, Graham BS. Illness severity, viral shedding, and antibody responses in infants hospitalized with bronchiolitis caused by respiratory syncytial virus. J Infect Dis 2002 April 15;185(8):1011-8.



Detection of respiratory viruses

CHAPTER 2

Increased detection of respiratory viruses with the use of real-time PCR in children and adults

Alma C. van de Pol
Anton M. van Loon
Tom F.W. Wolfs
Nicolaas J.G. Jansen
Monique Nijhuis
Els Klein Breteler
Rob Schuurman
John W.A. Rossen

J Clin Microbiol 2007;45:2260-2

Abstract

Respiratory samples (n = 267) from hospitalized patients with respiratory symptoms were tested by real-time PCR, viral culture, and direct immunofluorescence for respiratory syncytial virus, influenza virus, parainfluenza viruses, and adenoviruses. Compared with conventional diagnostic tests, real-time PCR increased the diagnostic yields for these viruses from 24% to 43% and from 3.5% to 36% for children and adults, respectively.

Introduction

Determining etiological diagnoses for patients admitted to the hospital with respiratory symptoms remains a clinical and laboratory challenge. Infections with respiratory syncytial virus (RSV), influenza virus (IV), parainfluenza virus (PIV), and adenovirus (AdV) are traditionally diagnosed using viral culture and direct immunofluorescence (DIF) tests. However, over the last 2 decades, nucleic acid amplification techniques, particularly real-time PCR, have become available as diagnostic tools. The aim of the present study was to determine the diagnostic yields of real-time PCR for RSV, IV, PIV and AdV in clinical practice, compared with those of traditional tests.

Methods

For this purpose, respiratory specimens from hospitalized pediatric and adult patients with respiratory symptoms were collected during one respiratory season (December 2004 through May 2005). Follow-up samples (taken within 4 weeks after a first sample) were excluded. Samples were divided into three aliquots. One aliquot of the samples from children was used for DIF assays to detect RSV A and B, IV A and B, PIV 1 to 3, and AdV using Imagen kits (DaKo, Glostrup, Denmark) according to the manufacturer's protocol. DIF was not performed for samples from adults, because it has been shown to have a very low sensitivity in an adult population (1). One aliquot of the samples was used for immediate viral culture of RSV, IV, PIV 1 to 3, and AdV on LLC-MK2, RD, R-HELA, and HEp-2C cells. Cultures were examined every other day for the development of a cytopathologic effect for 14 days, and positive cultures were confirmed by DIF with monoclonal antibodies specific for RSV A or B; IV A or B; PIV 1, 2, or 3; or AdV (DaKo, Glostrup, Denmark). Finally, an aliquot of the samples was used to extract viral

nucleic acids using the MagNA Pure LC total nucleic acid isolation kit (Roche Diagnostics, Basel, Switzerland) as described previously (2). Subsequently, cDNA was synthesized using MultiScribe reverse transcriptase and random hexamers (both from Applied Biosystems, Foster City, CA). Detection of viruses was performed using parallel real-time PCR assays for RSV A and B (3) and IV A and B as previously described (4;5). In addition, in-house real-time PCR methods were developed for the detection of PIV 1 to 4 and AdV using Primer Express (Applied Biosystems). Conserved target regions were identified using BLAST (www.ncbi.nlm.nih.gov/blast). Sequences of the primers and probes used are summarized in Table 1. Real-time PCR procedures were performed as described previously (2).

Table 1 Primers and probes used for real-time PCR detection

VIRUS(ES)	TARGET GENE	FORWARD PRIMER(S) (5'-3')	REVERSE PRIMER(S) (5'-3')	PROBE(S)[A]	REFERENCE
RSV A	Nucleocapsid	AGA TCA ACT TCT GTC ATC CAG CAA	TTC TGC ACA TCA TAA TTA GGA GTA TCA AT	FAM-CAC CAT CCA ACG GAG CAC AGG AGA T-TAMRA	(3)
RSV B	Nucleocapsid	AAG ATG CAA ATC ATA AAT TCA CAG GA	TGA TAT CCA GCA TCT TTA AGT ATC TTT ATA GTG	FAM-TTC CCT TCC TAA CCT GGA CAT AGC ATA TAA CAT ACC T-TAMRA	(3)
IV A	Matrix	AAG ACC AAT CCT GTC ACC TCT GA	CAA AGC GTC TAC GCT GCA GTC C	FAM-TTT GTG TTC ACG CTC ACC GT-TAMRA	(5)
IV B	Hemagglutinin	AAA TAC GGT GGA TTA AAC AAA AGC AA	CCA GCA ATA GCT CCG AAG AAA	FAM-CAC CCA TAT TGG GCA ATT TCC TAT GGC-TAMRA	(4)
PIV 1	Hemagglutinin-neuraminidase	TGA TTT AAA CCC GGT AAT TTC TCA T	CCT TGT TCC TGC AGC TAT TAC AGA	FAM-ACG ACA ACA GGA AAT C-MGB	
PIV 2	Hemagglutinin-neuraminidase	AGG ACT ATG AAA ACC ATT TAC CTA AGT GA	AAG CAA GTC TCA GTT CAG CTA GAT CA	FAM-ATC AAT CGC AAA AGC TGT TCA GTC ACT GCT ATA C-TAMRA	
PIV 3	Hemagglutinin-neuraminidase	TGA TGA AAG ATC AGA TTA TGC ATA TC	CCG GGA CAC CCA GTT GTG	FAM-TGG ACC AGG GAT ATA CTA CAA AGG CAA AAT AAT ATT TCT C-TAMRA	
PIV 4	Nucleocapsid	CAA AYG ATC CAC AGC AAA GAT TC	ATG TGG CCT GTA AGG AAA GCA	FAM-GTA TCA TCA TCT GCC AAA TCG GCA ATT AAA CA-TAMRA	
AVs	Hexon	TTT GAG GTG GAY CCM ATG GA	AGA ASG GSG TRC GCA GGT A	FAM-ACC ACG TCG AAA ACT TCG AA-MGB	
		TTT GAG GTY GAY CCC ATG GA	AGA ASG GTG TRC GCA GAT A	FAM-ACC ACG TCG AAA ACT TCA AA-MGB	
				FAM-ACA CCG CGG CGT CA-MGB	

[a] *FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine; MGB, minor groove binding.*

Results

A total of 267 samples from patients with respiratory symptoms were analyzed. Of these samples, 181 were from children (152 nasopharyngeal aspirate, 25 sputum, and 4 bronchoalveolar lavage specimens) and 86 from adults (77 nasal and/or throat swab, 6 bronchoalveolar lavage, and 3 sputum specimens). Twelve children and four adults contributed two samples.

For children, viral culture identified viruses in 25 (14%) samples, whereas DIF detected viruses in 35 (19%) samples. Thirty-eight (21%) samples were inadequate for analysis by DIF because of the absence of intact cells. The combination of viral culture and DIF detected viruses in 43 (24%) samples. A total of 78 (43%) samples were positive by real-time PCR; in these samples, 89 viruses were identified. Mixed viral infections were detected by real-time PCR only (seven [3.9%] double infections and two [1.1%] triple infections).

In adults, viral culture identified respiratory viruses in only three (3.5%) samples. Respiratory viruses were detected in 31 (36%) samples by real-time PCR; no mixed viral infections were detected.

The diagnostic yields of conventional methods compared to that of real-time PCR as the reference method are presented in Table 2. The sensitivities of conventional methods (culture and DIF) in diagnosing the pediatric population were 0.46 (confidence interval [CI], 0.38 to 0.53) for RSV, 0.44 (CI, 0.37 to 0.52) for IV, 0.63 (CI, 0.55 to 0.70) for PIV, and 0.24 (CI, 0.17 to 0.30) for AdV. The sensitivities of conventional methods (viral culture) for detection in adults appeared to be much lower, and were 0.00 (CI, 0.00 to 0.00) for RSV, 0.11 (CI, 0.04 to 0.18) for IV, 0.00 (CI, 0.00 to 0.00) for PIV, and 1.00 (CI, 1.00 to 1.00) for AdV. The specificities of conventional methods for the different respiratory viruses were 0.98 to 1.00 for children and 1.00 for adults.

Since the viral load in a sample can be relatively assessed by determining the cycle threshold (Ct) value, we compared the values for the samples that were culture/DIF positive with values for the samples that were culture/DIF negative. High Ct values are representative of a low viral load, while a low Ct value reflects a high viral load. For the pediatric population, the mean Ct value of samples positive for RSV by real-time PCR but in which RSV was not detected by conventional tests was 3.9 cycles higher (CI, 0.36 to 7.4) than that of positive samples subjected to detection by conventional tests ($p=0.03$). For AdV, the Ct value was 13 cycles higher (CI, 5.4 to 22; $p=0.003$). For IV, the trend was the same (5.7 cycles higher [CI, −0.25 to 12]; $p=0.06$). For PIVs, no difference was observed. For adults, this analysis could not be performed due to the small number of positive results by viral culture.

Table 2 Comparison of the results of conventional diagnostic tests and real-time PCR

PATIENT POPULATION	CONVENTIONAL TEST(S)	RESULT OF CONVENTIONAL TEST	NO. OF SPECIMENS WITH INDICATED PCR RESULT FOR:							
			RSV		IV		PIV		AdV	
			+	-	+	-	+	-	+	-
Children	Culture+DIF	+	21	1	8	1	5	0	4	3
		-	25	134	10	162	3	173	13	161
Adults	Culture	+	0	0	2	0	0	0	1	0
		-	10	76	16	68	2	84	0	85

Discussion

In the present study, we showed that real-time PCR for RSV, IV, PIV, and AdV increased the diagnostic yields for these viruses substantially in clinical practice, in comparison with those of conventional diagnostic tests.

Some limitations of our study deserve further discussion. First, there is no gold standard for the detection of respiratory viruses to which both conventional tests and real-time PCR can be compared. This is a problem encountered in all studies evaluating real-time PCR (6;7). Second, we did not use blind antibody fluorescence staining for viral culture and therefore may have missed positive cultures without cytopathogenic effects.

The findings of our study are in accordance with those of previous reports. Van Kraaij and colleagues identified RSV, IV, and PIV as present in 2% of 52 episodes of respiratory tract infection in adults after stem cell transplantation, whereas real-time PCR was positive for 12%, 4%, and 6% of the samples, respectively (8). Templeton and colleagues identified RSV, IV, and/or PIV in 19% of 358 respiratory samples by viral culture, compared with 24% of samples by real-time PCR (9). In the present study, we found an association between Ct values and conventional tests being positive. This was in agreement with the work of Bredius and colleagues, who found Ct values of 27 to 42 for samples positive only by real-time PCR versus Ct values of 18 to 22 for culture-positive samples (10).

Apart from being more sensitive than conventional methods, real-time PCR is also able to detect microorganisms that are difficult to culture, microorganisms for which no (commercial) DIF assay is available, or microorganisms that cannot be cultured at all. At our center, real-

time PCR is routinely performed for rhinovirus, coronavirus, human metapneumovirus, and *Mycoplasma pneumoniae*. These agents were found, respectively, in 47, 23, 13, and 5 pediatric samples and in 16, 6, 1, and 1 adult samples (unpublished data), increasing the detection rate further to 69% and 57% for children and adults, respectively.

In conclusion, real-time PCR considerably increases the diagnostic yields for respiratory viruses from patients admitted with respiratory symptoms within a clinically relevant time frame. This allows clinicians to initiate optimal patient management and to initiate adequate (future) use of antiviral therapy and optimal infection control.

Reference List

(1) Casiano-Colon AE, Hulbert BB, Mayer TK, Walsh EE, Falsey AR. Lack of sensitivity of rapid antigen tests for the diagnosis of respiratory syncytial virus infection in adults. J Clin Virol 2003 October;28(2):169-74.

(2) van de Pol AC, Wolfs TF, Jansen NJ, van Loon AM, Rossen JW. Diagnostic value of real-time polymerase chain reaction to detect viruses in young children admitted to the paediatric intensive care unit with lower respiratory tract infection. Crit Care 2006;10(2):R61.

(3) van Elden LJ, van Loon AM, van der BA, Hendriksen KA, Hoepelman AI, van Kraaij MG, Schipper P, Nijhuis M. Applicability of a real-time quantitative PCR assay for diagnosis of respiratory syncytial virus infection in immunocompromised adults. J Clin Microbiol 2003 September;41(9):4378-81.

(4) van Elden LJ, Nijhuis M, Schipper P, Schuurman R, van Loon AM. Simultaneous detection of influenza viruses A and B using real-time quantitative PCR. J Clin Microbiol 2001 January;39(1):196-200.

(5) Ward CL, Dempsey MH, Ring CJ, Kempson RE, Zhang L, Gor D, Snowden BW, Tisdale M. Design and performance testing of quantitative real time PCR assays for influenza A and B viral load measurement. J Clin Virol 2004 March;29(3):179-88.

(6) McAdam AJ. Discrepant analysis: how can we test a test? J Clin Microbiol 2000 June;38(6):2027-9.

(7) Murdoch DR. Molecular genetic methods in the diagnosis of lower respiratory tract infections. APMIS 2004 November;112(11-12):713-27.

(8) van Kraaij MG, van Elden LJ, van Loon AM, Hendriksen KA, Laterveer L, Dekker AW, Nijhuis M. Frequent detection of respiratory viruses in adult recipients of stem cell transplants with the use of real-time polymerase chain reaction, compared with viral culture. Clin Infect Dis 2005 March 1;40(5):662-9.

(9) Templeton KE, Scheltinga SA, Beersma MF, Kroes AC, Claas EC. Rapid and sensitive method using multiplex real-time PCR for diagnosis of infections by influenza a and influenza B viruses, respiratory syncytial virus, and parainfluenza viruses 1, 2, 3, and 4. J Clin Microbiol 2004 April;42(4):1564-9.
(10) Bredius RG, Templeton KE, Scheltinga SA, Claas EC, Kroes AC, Vossen JM. Prospective study of respiratory viral infections in pediatric hemopoietic stem cell transplantation patients. Pediatr Infect Dis J 2004 June;23(6):518-22.

CHAPTER 3

Diagnostic value of real-time PCR to detect viruses in PICU patients with lower respiratory tract infection

Alma C. van de Pol
Tom F.W. Wolfs
Nicolaas J.G. Jansen
Anton M. van Loon
John W.A. Rossen

Critical Care 2006;10:R61

Abstract

Introduction

The etiology of lower respiratory tract infections in young children admitted to the pediatric intensive care unit (PICU) is often difficult to establish. However, most infections are believed to be caused by respiratory viruses. A diagnostic study was performed to compare conventional viral tests with the recently developed real-time PCR technique.

Methods

Samples from children aged under 5 years presenting to a tertiary PICU suspected of having a lower respiratory tract infection were tested using conventional methods (viral culture and immunofluorescence) and real-time PCR during the winter season from December 2004 to May 2005. Conventional methods were used to check for respiratory syncytial virus, influenza virus, parainfluenza virus 1–3, rhinovirus and adenovirus. Real-time PCR was used to test for respiratory syncytial virus, influenza virus, parainfluenza virus 1–4, rhinovirus, adenovirus, human coronavirus OC43, NL63, and 229E, human metapneumovirus, *Mycoplasma pneumoniae* and *Chlamydia pneumoniae*.

Results

A total of 23 patients were included, of whom 11 (48%) were positive for a respiratory virus by conventional methods. Real-time PCR confirmed all of these positive results. In addition, real-time PCR identified 22 more viruses in 11 patients, yielding a total of 22 (96%) patients with a positive sample. More than one virus was detected in eight (35%) children.

Conclusion

Real-time PCR for respiratory viruses was found to be a sensitive and reliable method in PICU patients with lower respiratory tract infection, increasing the diagnostic yield twofold compared to conventional methods.

Introduction

Lower respiratory tract infections (LRTIs) cause significant hospitalization among children under 5 years old (1;2). Although these infections are usually relatively mild, some children develop respiratory failure, necessitating mechanical ventilation and admission to the pediatric intensive care unit (PICU). The etiology of (severe) LRTI is often difficult to establish. However, the majority of LRTIs in this young age group is believed to be caused by respiratory viruses. For the detection of respiratory viruses two conventional methods are commonly used: viral culture and direct immunofluorescence (DIF) assays. In addition, a third method based on molecular techniques has now become available, namely (real-time) PCR. Specifically, the real-time PCR format provides rapid results, within a clinically relevant period of time. It allows quantitative virus detection, and no post-PCR processing needs to be performed. Moreover, compared with standard format PCR, the risk for contamination is strongly reduced with real-time PCR, rendering false-positive results with real-time PCR highly unlikely (3).

Currently, there are no guidelines regarding which viral tests are appropriate for young children with LRTI in the PICU (4). Conventional detection methods have several disadvantages compared with (real-time) PCR. Viral culture has been considered the gold standard for the detection of respiratory viruses, but its limitation is that it can only detect a small number. Furthermore, the yield of viral culture depends on the quality of sampling, the correct transport and storage of samples, and the type of cells used. In addition, the technique has a turnaround time of several days to weeks, and is therefore unable to guide initial patient management. The results of DIF assays are available more rapidly, and this has been shown to improve patient outcome with less antibiotic use and shorter hospital stay (5-7). Unfortunately, the DIF assay is less sensitive than culture for the detection of certain pathogens.

PCR has been shown to be more sensitive than conventional techniques and is fast (8-11). Additional advantages of PCR include its ability to identify multiple viruses simultaneously, and to detect viral and atypical pathogens that cannot be cultured or for which no DIF is commercially available (for example, coronavirus, human metapneumovirus [hMPV], *Chlamydia pneumoniae* and *Mycoplasma pneumoniae*). Rapid and sensitive testing for a broad range of respiratory viruses is vital in the PICU, and it can improve our understanding of severe viral respiratory infections. Furthermore, it can be used to guide cohorting strategies that may protect other critically ill children and to guide initial therapy. Finally, evaluation of viral tests may be of future importance, when antiviral therapy becomes more widely available.

In the present study conventional methods (viral culture and DIF) were compared with real-time PCR for their ability to detect respiratory viruses in young children with LRTI admitted to the PICU. Secondary objectives were to describe the presence of viral pathogens and bacterial infection in LRTI patients in the PICU.

Methods

Study population

Children aged under 5 years admitted to the PICU of the Wilhelmina Children's Hospital with LRTI were enrolled during one winter season from December 2004 to May 2005. Wilhelmina Children's Hospital is a tertiary university hospital with a 14 bed PICU facility, and serves as a referral centre for the central part of The Netherlands.

Patients were eligible if they had an admission diagnosis of (probable) bronchiolitis, (probable) pneumonia, or respiratory failure. Exclusion criteria were a primary cardiac or central origin of respiratory failure, and overt sepsis at the time of admission. The study described here was conducted as part of normal patient care; therefore, according to the Medical Ethical Research Council of our institution, there was no need for patient consent/ethical approval.

Samples

Sputa and/or nasopharyngeal aspirates were obtained from all eligible patients for viral testing by viral culture, DIF and real-time PCR assays. In addition, sputum and blood samples (when available) were cultured and processed in accordance with standard microbiological procedures. Sputum samples were taken in a standardized manner, through sterile transtracheal aspiration in intubated children.

Conventional virus detection

Some of the material obtained was used for immediate viral culture of RSV, influenza virus, parainfluenza virus (PIV) 1–3, picornavirus, adenovirus and herpes virus on LCMK2, RD, R-HELA and HEP-2C cells. Cultures were examined twice weekly for the development of a cytopathological effect. In positive cultures, virus was identified by immunofluorescence with monoclonal antibodies to RSV types A and B, influenza virus A and B, PIV 1–3 and adenovirus (DaKo, Glostrup, Denmark). Rhinovirus was identified using acid lability tests.

Some of the material was subjected to DIF assays to detect respiratory syncytial virus (RSV), influenza viruses, PIV 1–3 and adenoviruses using Imagen kits (DaKo), in accordance with the manufacturer's recommended protocol. The remaining material was used for real-time PCR testing.

Real-time PCR

Nucleic acids were extracted using the total nucleic acid protocol with the MagNA pure LC nucleic acid isolation system (Roche Diagnostics, Basel, Switzerland). Each sample was eluted in 200 µl buffer, which was sufficient for all real-time PCR analyses. cDNA was synthesized by using MultiScribe reverse transcriptase (RT) and random hexamers (both from Applied Biosystems, Foster City, CA, USA). Each 200 µl reaction mixture contained 80 µl of eluted RNA, 20 µl of 10 × RT buffer, 5.5 mmol/l $MgCl_2$, 500 µmol/l of each deoxynucleoside triphosphate, 2.5 µmol/l random hexamer and 0.4 U of RNase inhibitor per µl (all from Applied Biosystems). After incubation for 10 minutes at 25°C, RT was carried out for 30 minutes at 48°C, followed by RT inactivation for 5 minutes at 95°C.

Detection of viral and atypical pathogens was performed in parallel, using real-time PCR assays specific for the following: RSV A and B, influenza virus A and B, PIV 1–4, rhinovirus, adenovirus, human coronavirus OC43, NL63 and 229E, hMPV, *Mycoplasma pneumoniae* and *Chlamydia pneumoniae*. Real-time PCR procedures were performed as described previously (12-14).

Briefly, samples were assayed in duplicate in a 25 µl reaction mixture containing 10 µl (c)DNA, 12.5 µl 2 × TaqMan Universal PCR Master Mix (Applied Biosystems), 300–900 nmol/l of the forward and reverse primers and 75–200 nmol/l of each of the probes. All samples had been spiked before extraction with an internal control virus (murine encephalomyocarditis virus [RNA virus] and phocine herpes virus [DNA virus]) to monitor for efficient extraction and amplification, essentially as described previously (15).

Clinical data collection and analysis

Each patient's medical records were reviewed for clinical data. Demographic data and the following clinical characteristics/outcomes were extracted and entered into standardized forms: gestational age, underlying disease, length of PICU stay, need for mechanical ventilation and death. Five criteria were used to classify the presence of bacterial infection at admission or during the PICU stay: new infiltrate on the chest radiograph, increased need for supplemental oxygen, fever (temperature of 38.5°C or greater), increased infectious parameters (C-reactive protein >40 mg/l and/or white blood cell count >15 × 109/l) and positive bacterial culture (blood or sputum). For the purpose of this study, "no bacterial infection", "possible bacterial infection" and "proven bacterial infection" were defined as the presence of fewer than two, two to three, and more than three of the above criteria, respectively.

For the analysis of continuous variables, an unpaired t test was used to compare means. Categorical variables were analyzed using a two-tailed Fisher's exact test.

Results

Twenty-six PICU patients met the inclusion criteria. One patient was admitted with a positive RSV test result from an outside hospital, and no further testing was performed at our institution. Another patient diagnosed with a bacterial pneumonia was subjected to no virus tests, and insufficient material was obtained from a third patient to perform all respiratory tests. Consequently, 23 patients were included in the subsequent analysis.

The median age of the patients was 2.6 months, and the majority was referred from an outside hospital (Table 1). The PICU patients formed a heterogeneous group, with many patients having underlying conditions resulting from preterm birth or birth defects. Patients stayed in the PICU for a median of 10 days; 20 (87%) patients needed mechanical ventilation. Two patients in the study group died. One child suffered from bronchiolitis with progressive bronchospasms, which in the end could not be controlled. The second child had complex underlying conditions; after eliminating all treatable causes (including LRTI) the patient died from central hypoventilation.

Viral culture identified a respiratory virus in six PICU patients; RSV and adenovirus were detected in four and two patients, respectively (Table 2). The DIF assay appeared to be more sensitive to detect respiratory viruses than viral culture in our population, identifying a virus in 11 (48%) patients, including the six patients diagnosed by viral culture.

Real-time PCR detected a total of 33 respiratory viruses in 22 (96%) patients. All positive results found by conventional techniques were confirmed by real-time PCR. RSV was the single most common respiratory virus found and was detected in 16 (70%) patients. In addition, rhinovirus was found in six (26%) patients, and influenza virus, adenovirus and coronavirus were each detected in three (13%) children. PIV-3 and hMPV were both found in one patient (4%). PIV-2 and PIV-4 and the atypical pathogens *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* could not be detected in any of the LRTI patients.

In eight (35%) children more than one virus was detected. Six children had a dual infection, one had a triple, and one had a quadruple respiratory virus infection. RSV was detected in seven of the eight children infected with multiple viruses, including all three patients infected with a coronavirus. Of the 14 children with a single infection, nine had RSV. The other five patients had an infection with rhinovirus (n = 2), influenza virus (n = 1), adenovirus (n = 1), or hMPV (n = 1).

No statistical differences were found between children with a single and multiple virus infection with respect to age, sex, gestational age, underlying disease, length of PICU stay, mechanical ventilation and the presence of bacterial infection.

Bacterial cultures were performed in 14 patients. In eight (57%) patients bacterial pathogens were identified (most commonly *Streptococcus pneumoniae*, *Moraxella catarrhalis, Haemophilus influenzae* and *Staphylococcus aureus*). The details for each patient regarding viral and bacterial infections, including length of stay, are presented in Table 3. According to the predefined classification, six (26%) children in the study group had proven, 11 (48%) had possible and six (26%) had no bacterial infection. Of the six patients with a proven bacterial infection, two infections developed after more than 48 hours of mechanical ventilation. Overall, 19 (83%) patients received antibiotics during their stay on the PICU for a median of 7 days. Patients with a proven bacterial infection stayed in the PICU for a mean of 18 days versus 9 days for patients with possible or no bacterial infection (p = 0.026).

Table 1 Demographic and clinical characteristics of children with lower respiratory tract infections on admission to the PICU

CHARACTERISTICS	VALUE
Demographics	
Age, median months (range)	2.6 (0.5-26.5)
Male	10 (43%)
Admission from outside hospital	20 (87%)
Underlying conditions	
Preterm birth (<37 weeks)	11 (48%)
Underlying disease	11 (48%)
Pulmonary	3
Cardiac	4
Other	5
Severity	
PICU stay (days; median [range])	10 (2-33)
Mechanically ventilated at PICU	20 (87%)
Death due to LRTI	1

A total of 23 patients were included in the study. LRTI, lower respiratory tract infections; PICU, pediatric intensive care unit.

Table 2 Viruses identified by conventional methods and real-time PCR

PATHOGEN	VIRAL CULTURE (N=21)	IMMUNOFLUORESCENCE (N=22)	REAL-TIME PCR (N=23)
RSV A/B	4	7	16 (9)
Influenza virus A/B	0	2	3 (1)
Rhinovirus	0		6 (2)
Adenovirus	2	2	3 (1)
Coronavirus OC43, 229E, NL63			3
hMPV			1 (1)
PIV 1/3	0	0	1
PIV 2/4	0	0	0
Chlamydia pneumoniae			0
Mycoplasma pneumoniae			0
Indeterminate		4	
Total positives	6[a]	11 [a]	33

Numbers in parentheses indicate single infections. [a]Single infections. hMPV, human metapneumovirus; PCR, polymerase chain reaction; PIV, parainfluenza virus; RSV, respiratory syncytial virus.

Table 3 Viral and bacterial pathogens and bacterial infection specified per patient

PATIENT	AGE (MONTHS)	UNDERLYING DISEASE	ICU STAY (DAYS)	BACTERIAL INFECTION	BACTERIAL CULTURE	VIRAL CULTURE	IMMUNO-FLUORES-CENCE	REAL-TIME PCR
1	1.7	Preterm birth	14	Possible	*S. pneumoniae, M. catarrhalis, H. influenzae*[c]			RSV
2	1.6	Preterm birth, long-term oxygen	33	Proven	*S. pneumoniae, S. aureus*[c]		RSV	RSV
3	26.5	Preterm birth, BPD	15	No				RSV

4a	0.8	Spina bifida, Arnold Chiari type II malformation	6	No			indeterminate	RSV, Rhinovirus
5	0.8		18	Possible	*M. catarrhalis*[c]		indeterminate	RSV
6	2.6		4	Possible			RSV	RSV
7	2.3		8	No				
8	2.7	Preterm birth, IVH	16	Proven	*H. influenza*[d]	RSV	RSV	RSV
9	2.4	Multiple congenital disorders, microcephaly	3	No				Parainfluenzavirus 3, Rhinovirus
10	1.2	Preterm birth	13	Possible		RSV	RSV	RSV
11	8	Asthma	24	Proven	*K. oxytoca, H. influenza*[c]	Adenovirus	Adenovirus	RSV, Adenovirus, Coronavirus, Rhinovirus
12	3.2	Preterm birth, s/p ASD, VSD, PDA	15	Proven	*S. aureus*[c]		Influenzavirus	RSV, Influenzavirus
13	2.6		10	Possible			RSV	RSV, Influenzavirus, Adenovirus
14	11.4	Preterm birth	4	Possible		Adenovirus	Adenovirus	Adenovirus
15b	2.3		7	Possible	*S. pneumoniae, M. catarrhalis*[c]		Influenzavirus	Influenzavirus
16	8.8	ASD, VSD	10	Possible		RSV	RSV	RSV
17	0.5		2	No		RSV	RSV	RSV, rhinovirus
18	2.6		11	Possible			indeterminate	RSV, coronavirus
19	4.4	Preterm birth, s/p NEC, colostoma	9	Possible			indeterminate	Rhinovirus

Table 3 continuation

PATIENT	AGE (MONTHS)	UNDERLYING DISEASE	ICU STAY (DAYS)	BACTERIAL INFECTION	BACTERIAL CULTURE	VIRAL CULTURE	IMMUNO-FLUORESCENCE	REAL-TIME PCR
20	4.9	Preterm birth	12	Proven	*S. pneumoniae, M. catarrhalis, H. influenza*[c]			human Metapneumovirus
21	2.1	Preterm birth	10	Possible				RSV, Coronavirus
22	2.1	Preterm birth, ASD, PDA, TI, PHT	10	No				RSV
23	5.8		10	Proven				Rhinovirus

[a] *Died, cause: LRTI.* [b] *Died, cause: underlying disease.* [c] *Sputum culture.* [d] *Blood culture.*
ICU, intensive care unit; RSV, respiratory syncytial virus; BPD, bronchopulmonary dysplasia; IVH, intraventricular hemorrhage; s/p, status post; ASD, atrial septal defect; VSD, ventricular septal defect; PDA, patent ductus arteriosus; NEC, necrotizing enterocolitis; TI, tricuspid insufficiency; PHT, pulmonary hypertension.

Discussion

In the present study conventional methods (viral culture and DIF) were compared with real-time PCR for their ability to detect respiratory viruses in young children admitted to the PICU with LRTI. The use of real-time PCR increased the diagnostic yield from 48% to 96%, and all viruses found by conventional methods were confirmed by real-time PCR. Whereas with conventional methods no double infections were found, real-time PCR revealed multiple virus infections in 35% of patients.

The high diagnostic yield of PCR compared with conventional techniques is in agreement with the findings of others. When conducting a Medline search (1966 to September 2005) with the search terms LRTI, PCR, and RSV or respiratory viruses, we identified 11 hospital-based pediatric studies that compared PCR with conventional techniques for more than two respiratory viruses (8-11;16-22). PCR was found to have excellent sensitivity in all studies, if conventional methods were considered the reference standard (0.91–1.00). In summary, these studies showed that PCR increased the diagnostic yield by up to 25% as compared with conventional techniques.

However, most studies selected patients on the basis of respiratory test results, rather than selecting patients by admission diagnosis. Exceptions include the studies conducted by Weinberg and coworkers (22) and Jennings and colleagues (9). Weinberg and coworkers (22) tested samples from 668 hospitalized children and selected patients on admission diagnoses by the New Vaccine Surveillance Network. PCR assays were used to test for RSV, influenza viruses and PIV 1–3. With viral culture 89 (13%) specimens were positive, as compared with 185 (28%) positive specimens with PCR. Jennings and colleagues (9) enrolled 75 hospitalized children using a case definition of acute respiratory infection, and performed viral diagnostics for a broad range of viruses. PCR identified 39 additional viruses, adding up to a total of 87 viruses found in 65 (87%) children (9).

The clinical relevance of detection of respiratory viruses such as rhinovirus and coronavirus is a matter of debate. Rhinovirus (23) and coronavirus (14) are well recognized as causes of the common cold. On the other hand, there is increasing evidence that they are also important causes of severe lower respiratory disease. Rhinovirus infections have been reported in elderly adults with LRTI or pneumonia (24;25) and in immunocompromised patients (26;27). Coronavirus infection has also been reported in cases of LRTI (14;24;28;29). Real-time PCR will provide insight into the role played by such viruses in the etiology of LRTI.

The high yield of PCR compared with conventional techniques could theoretically be the result of contamination. However, with using real-time PCR the likelihood of false-positive results caused by carry-over contamination is reduced to a minimum. This is achieved by the use of modified nucleotides (dUTP) and uracil-DNA glycosylase (UNG) for control of contamination in the PCR-based amplification of (c) DNA as well as the closed-tube detection system (3). In addition, during the procedures of DNA/RNA isolation and amplification, several negative controls are included to monitor for possible false positive findings.

An additional advantage of real-time PCR over standard-format PCR is that it allows high-throughput screening of patient samples for the presence of many different pathogens. Our real-time PCR assays included testing not only for RSV, influenza virus and PIV, but also for rhinovirus, adenovirus, the recently discovered hMPV (30), coronavirus (OC43, 229E and the newly identified coronavirus NL63 (31) and the atypical pathogens *Mycoplasma pneumoniae* and *Chlamydia pneumoniae*. Thus, real-time PCR allows for maximal detection of multiple viral and atypical infections in children with LRTI, with a negligible risk for false positive results.

In the PICU patients studied, we found a high prevalence of multiple viral infections (35%), including triple and even quadruple infections. In previous studies the detection of multiple infections varied considerably, ranging from 0.6% to 27%. A low prevalence was found in studies using PCR for RSV, influenza viruses and PIVs only (18;22). In contrast, a high prevalence

was found in studies that included a broad range of respiratory viruses (including rhinovirus, adenovirus, coronavirus and hMPV) (9). Interestingly, in the latter study most coronaviruses were identified as part of multiple virus infections (three out of four), which is in accordance with our findings.

The prevalence of possible or proven bacterial infection in our study group was high (74%). The fact that almost half of the children were diagnosed with a possible bacterial infection indicates the difficulty of precluding this diagnosis. In addition, previous studies showed that viral infection may pave the way for bacterial infection (32). The only study that compared viral diagnostic methods in a similar population of PICU patients did not report on the prevalence of bacterial infections, and so we cannot compare this finding with those from other studies (16). In contrast to the high prevalence of possible or proven bacterial infection found in our study group, we did not demonstrate infections with atypical pathogens such as *Mycoplasma pneumoniae* and *Chlamydia pneumoniae*. It can be speculated that atypical pathogens do not play an important role in children with severe LRTI.

The small study group included in our study represents a limitation; it did not allow us to find an association between clinical characteristics and multiple infections. Furthermore, the samples that were negative by conventional methods and positive by real-time PCR could not be confirmed using a true gold standard because such a standard for respiratory viruses does not exist. This is a problem encountered in all studies of respiratory viruses.

The finding that real-time PCR increases the yield of viral diagnoses for PICU patients with LRTI has implications for clinical practice. The rapidity and sensitivity of real-time PCR test results can help the clinician to initiate appropriate cohorting strategies to prevent other critically ill children from nosocomial viral infections. A reliable and rapid viral test result can also be taken into account when prescribing or discontinuing antibiotic treatment. Our study was not designed to determine when antibiotic treatment for LRTI in the PICU is warranted. The high percentage of possible or proven bacterial infections in our viral LRTI patients indicates that antibiotic treatment poses a dilemma in the PICU. The clinical impact of the high prevalence of multiple virus infections detected in our PICU remains unclear because they were not associated with different clinical characteristics. Further research with larger numbers of patients and age-matched control groups is needed to determine the real clinical impact of multiple infections and to determine whether the use of real-time PCR prevents unnecessary antibiotic treatment. However, real-time PCR offers a rapid, sensitive and highly reliable new technique that may improve our understanding of the epidemiology of severe LRTIs.

In conclusion, real-time PCR for a broad range of respiratory viruses was found to be highly sensitive in children with severe lower respiratory tract infection in the PICU. In addition,

real-time PCR increased the diagnostic yield of positive samples by twofold compared with conventional methods (viral culture and DIF). Whereas conventional methods identified no multiple infections, real-time PCR found the prevalence of multiple infections to be 35%. Because real-time PCR is a rapid and sensitive technique, it is able to guide initial patient cohorting strategies and therapy in the PICU.

Reference List

(1) Henrickson KJ, Hoover S, Kehl KS, Hua W. National disease burden of respiratory viruses detected in children by polymerase chain reaction. Pediatr Infect Dis J 2004 January;23 (1 Suppl):S11-S18.

(2) Shay DK, Holman RC, Newman RD, Liu LL, Stout JW, Anderson LJ. Bronchiolitis-associated hospitalizations among US children, 1980-1996. JAMA 1999 October 20;282(15):1440-6.

(3) Pang J, Modlin J, Yolken R. Use of modified nucleotides and uracil-DNA glycosylase (UNG) for the control of contamination in the PCR-based amplification of RNA. Mol Cell Probes 1992 June;6(3):251-6.

(4) Henrickson KJ. Advances in the laboratory diagnosis of viral respiratory disease. Pediatr Infect Dis J 2004 January;23(1 Suppl):S6-10.

(5) Adcock PM, Stout GG, Hauck MA, Marshall GS. Effect of rapid viral diagnosis on the management of children hospitalized with lower respiratory tract infection. Pediatr Infect Dis J 1997 September;16(9):842-6.

(6) Barenfanger J, Drake C, Leon N, Mueller T, Troutt T. Clinical and financial benefits of rapid detection of respiratory viruses: an outcomes study. J Clin Microbiol 2000 August;38(8):2824-8.

(7) Woo PC, Chiu SS, Seto WH, Peiris M. Cost-effectiveness of rapid diagnosis of viral respiratory tract infections in pediatric patients. J Clin Microbiol 1997 June;35(6):1579-81.

(8) Gruteke P, Glas AS, Dierdorp M, Vreede WB, Pilon JW, Bruisten SM. Practical implementation of a multiplex PCR for acute respiratory tract infections in children. J Clin Microbiol 2004 December;42(12):5596-603.

(9) Jennings LC, Anderson TP, Werno AM, Beynon KA, Murdoch DR. Viral etiology of acute respiratory tract infections in children presenting to hospital: role of polymerase chain reaction and demonstration of multiple infections. Pediatr Infect Dis J 2004 November;23(11):1003-7.

(10) Kehl SC, Henrickson KJ, Hua W, Fan J. Evaluation of the Hexaplex assay for detection of respiratory viruses in children. J Clin Microbiol 2001 May;39(5):1696-701.

(11) Syrmis MW, Whiley DM, Thomas M, Mackay IM, Williamson J, Siebert DJ, Nissen MD, Sloots TP. A sensitive, specific, and cost-effective multiplex reverse transcriptase-PCR assay for the detection of seven common respiratory viruses in respiratory samples. J Mol Diagn 2004 May;6(2):125-31.
(12) van Elden LJ, Nijhuis M, Schipper P, Schuurman R, van Loon AM. Simultaneous detection of influenza viruses A and B using real-time quantitative PCR. J Clin Microbiol 2001 January;39(1):196-200.
(13) van Elden LJ, van Loon AM, van der BA, Hendriksen KA, Hoepelman AI, van Kraaij MG, Schipper P, Nijhuis M. Applicability of a real-time quantitative PCR assay for diagnosis of respiratory syncytial virus infection in immunocompromised adults. J Clin Microbiol 2003 September;41(9):4378-81.
(14) van Elden LJ, van Loon AM, van AF, Hendriksen KA, Hoepelman AI, van Kraaij MG, Oosterheert JJ, Schipper P, Schuurman R, Nijhuis M. Frequent detection of human coronaviruses in clinical specimens from patients with respiratory tract infection by use of a novel real-time reverse-transcriptase polymerase chain reaction. J Infect Dis 2004 February 15;189(4):652-7.
(15) van Doornum GJ, Guldemeester J, Osterhaus AD, Niesters HG. Diagnosing herpesvirus infections by real-time amplification and rapid culture. J Clin Microbiol 2003 February;41(2):576-80.
(16) Akhtar N, Ni J, Stromberg D, Rosenthal GL, Bowles NE, Towbin JA. Tracheal aspirate as a substrate for polymerase chain reaction detection of viral genome in childhood pneumonia and myocarditis. Circulation 1999 April 20;99(15):2011-8.
(17) Bellau-Pujol S, Vabret A, Legrand L, Dina J, Gouarin S, Petitjean-Lecherbonnier J, Pozzetto B, Ginevra C, Freymuth F. Development of three multiplex RT-PCR assays for the detection of 12 respiratory RNA viruses. J Virol Methods 2005 June;126(1-2):53-63.
(18) Erdman DD, Weinberg GA, Edwards KM, Walker FJ, Anderson BC, Winter J, Gonzalez M, Anderson LJ. GeneScan reverse transcription-PCR assay for detection of six common respiratory viruses in young children hospitalized with acute respiratory illness. J Clin Microbiol 2003 September;41(9):4298-303.
(19) Fan J, Henrickson KJ, Savatski LL. Rapid simultaneous diagnosis of infections with respiratory syncytial viruses A and B, influenza viruses A and B, and human parainfluenza virus types 1, 2, and 3 by multiplex quantitative reverse transcription-polymerase chain reaction-enzyme hybridization assay (Hexaplex). Clin Infect Dis 1998 June;26(6):1397-402.
(20) Freymuth F, Vabret A, Galateau-Salle F, Ferey J, Eugene G, Petitjean J, Gennetay E, Brouard J, Jokik M, Duhamel JF, Guillois B. Detection of respiratory syncytial virus, parainfluenzavirus 3, adenovirus and rhinovirus sequences in respiratory tract of infants by polymerase chain reaction and hybridization. Clin Diagn Virol 1997 May;8(1):31-40.

(21) Gilbert LL, Dakhama A, Bone BM, Thomas EE, Hegele RG. Diagnosis of viral respiratory tract infections in children by using a reverse transcription-PCR panel. J Clin Microbiol 1996 January;34(1):140-3.
(22) Weinberg GA, Erdman DD, Edwards KM, Hall CB, Walker FJ, Griffin MR, Schwartz B. Superiority of reverse-transcription polymerase chain reaction to conventional viral culture in the diagnosis of acute respiratory tract infections in children. J Infect Dis 2004 February 15;189(4):706-10.

CHAPTER 4

Conventional versus molecular viral tests for respiratory viruses: a systematic review

Alma C. van de Pol
Marieke M. van der Zalm
Nicolaas J.G. Jansen
Cornelis K. van der Ent
Anton M. van Loon
Jan L.L. Kimpen
John W.A. Rossen
Maroeska M. Rovers
Tom F.W. Wolfs

Submitted

Abstract

Objective

From a laboratory perspective, conventional respiratory viral tests (antigen detection by immunofluorescence and viral culture) are assumed to be less sensitive than nucleic acid amplification tests (NATs). We systematically reviewed the literature to estimate the diagnostic accuracy of conventional tests compared to NATs as the reference standard, when studied in the pediatric clinical setting.

Design

We searched Medline, Embase, and bibliographies of relevant articles for studies that compared conventional tests and NATs for the diagnosis of respiratory viruses. Descriptions of study methodology were critically appraised using the QUADAS criteria for quality assessment.

Setting and Patients

We included studies with infants or children admitted to the hospital with respiratory symptoms.

Interventions

None.

Main outcome measures

Sensitivity and specificity of conventional viral tests compared to NATs.

Results

A total of 33 publications (10297 patients) were included in the analysis. Viral diagnostic approaches varied considerably. The majority of studies reported less than 70% of QUADAS

items. Important flaws comprised a lack of clear criteria for patient selection and the absence of blinding. Study results could not be pooled due to heterogeneity. The sensitivity of conventional tests varied between 0.00 and 1.00, whereas the specificity was mostly higher than 0.90.

Conclusions

Compared to NATs as the reference standard, the sensitivity of conventional tests varied considerably between studies. Because clinical studies used a large variety of viral test approaches, the anticipated inferiority of conventional viral tests could not be confirmed. International standards are needed to assure efficient viral diagnosis and patient management.

Introduction

Viral respiratory tract infections are a major public health problem and a common cause of hospitalization in infants and children (1-4). Over the past decades techniques for diagnosing respiratory syncytial virus (RSV), influenza viruses (IV), parainfluenza viruses 1-3 (PIVs), and adenoviruses (AdV) have advanced substantially. Traditionally, two types of conventional assays are used: direct antigen detection by immunofluorescence (IF) and viral culture. Currently, nucleic acid amplification tests (NATs), particularly polymerase chain reaction (PCR) assays, are increasingly being performed.

Efficient detection of respiratory viruses is important for optimal patient management and infection control, especially since the possibilities for antiviral drugs increase. Conventional tests have disadvantages, such as the fact that results are easily influenced by adverse transportation and storage conditions. Furthermore, they are not available for all known respiratory viruses (e.g. human coronavirus and human metapneumovirus can only be readily detected by NATs). Unfortunately, no golden standard exists for the detection of viruses. In the absence of a golden standard, NATs are generally believed to be the reference method against which conventional tests should be compared (5-8). In laboratory papers conventional tests have been shown to have a low sensitivity in comparison with NATs. However, these papers do not report clinical data such as patients' symptoms and inpatient or outpatient status (9-11). Therefore, the impact of the presumed inferior sensitivity of conventional tests in daily clinical practice remains unclear. Since conventional tests also have advantages such as low costs and straight forward technology, confirmation of the inferiority of conventional tests in well performed clinical studies is warranted before changing to NATs.

The present study aims to determine the diagnostic value of conventional tests (IF/viral culture), compared to NATs as the reference method, in clinical studies with hospitalized infants and children with respiratory symptoms.

Methods

Search strategy and selection criteria

We identified studies by searching Medline and Embase from inception through October 2008 using the following search terms: (1) "respiratory (tract) infection" "respiratory (tract) disease", "respiratory (tract) illness", "pneumonia", "bronchiolitis", "(infection with) respiratory viruses", and "respiratory symptoms"; (2) "polymerase chain reaction"; (3) "respiratory syncytial virus", "influenza virus", "parainfluenza viruses", "adenoviruses", and "RNA viruses"; (4) "infant", "child", and "p(a)ediatric". Predefined inclusion criteria were: 1) comparison of IF or viral culture to NATs for the diagnosis of RSV, IV, PIV, or AdV; 2) inclusion of infants or children admitted to a hospital with respiratory symptoms; 3) language: English, Spanish, French, German or Dutch (12). Authors of relevant articles were contacted for additional information when necessary. Two reviewers (AvdP and MvdZ) independently screened titles, abstracts, and full texts for eligibility. Overall agreement was calculated. Disagreement among reviewers was resolved by discussion and third party consultation as needed.

Quality assessment and data abstraction

The methodological quality of the studies was evaluated using the Quality Assessment of Diagnostic Accuracy Studies (QUADAS) checklist (13;14). The following items were scored as Yes, No or Unclear: (1) clear selection criteria; (2) well described conventional test; (3) well described NAT; (4) conventional test interpreted without knowledge of NAT result; (5) NAT interpreted without knowledge of conventional test results; (6) inadequate test results reported; (7) withdrawals from the study were explained. The remaining 7 items from the 14-item complete QUADAS tool were all scored as Yes, since these pertained to inclusion criteria for our systematic review.

The study population was classified for respiratory illness (lower respiratory tract infection, upper respiratory tract infection, "acute respiratory infection", a (combination of upper and lower) respiratory infection, or "respiratory symptoms") and age (<1 year or "infants", <5 year,

<18 year or "children"). NATs were classified into four categories: (a) PCR identification with gel detection, (b) PCR identification with a hybridization assay, (c) nested PCR, (d) real-time PCR or Nucleic Acid Sequence-Based Amplification (NASBA). If one publication evaluated two different assays or two different specimen types, results from each assay or specimen type were considered as an individual study. Detailed characteristics of viral tests were abstracted and 2x2 tables were constructed from raw data presented in reports. Both quality assessment and data abstraction were performed independently by two reviewers (AvdP and MvdZ).

Data-analysis

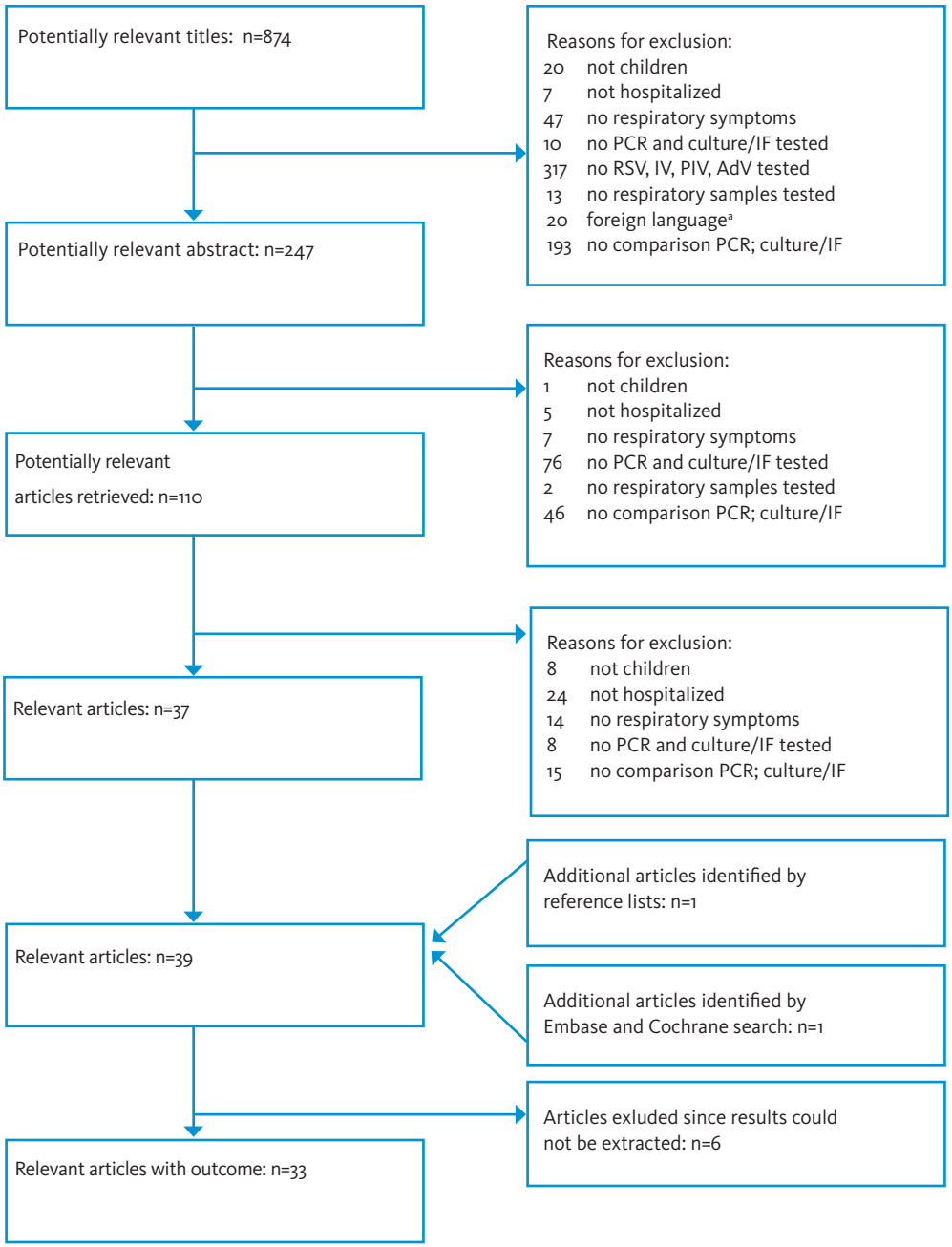
From each 2x2 table we computed sensitivity and specificity and their corresponding 95% confidence intervals (CIs) using MetaDisc (15). NATs were always regarded as the reference method against which conventional test sensitivity was calculated, regardless of the type of analyses reported in individual articles. To assess heterogeneity, I2 statistics were computed (16). I2 is derived from Cochran's Q statistics. It measures the extent of inconsistency among study results, and the outcome is interpreted as the percentage of total variation across studies that is due to heterogeneity rather than chance. A value of 0% indicates that all variability in results is due to chance and that none is due to heterogeneity. Larger values show that most of the variability is due to heterogeneity, rather than chance. When I2 was >25% (i.e., >25% of the variability is due to heterogeneity), no pooled sensitivity and specificity estimates were calculated. To explore sources of heterogeneity, the following pre-planned subgroups were analyzed separately: respiratory illness category, age category, and PCR type category. Publication bias was evaluated by funnel plots (17).

Results

Selection of studies

Figure 1 shows the flow of studies through the review. Thirty-nine publications met the inclusion criteria. For 6 studies, no relevant outcomes could be abstracted due to incomplete reporting(18-23), i.e. 33 publications were included in this systematic review. Five publication reported results of two different PCR assays (24-28) and one study evaluated two different specimen types (29), hence 39 studies were available for data-analysis. Overall agreement between the two investigators that performed the selection of relevant studies was 96%.

Figure 1 Inclusion of relevant articles



[a] *languages other than English, Spanish, French, German or Dutch*

Study characteristics

Methodology

Table 1 provides details of the 33 included publications. The description of study methodology was generally poor: 23 of the 33 studies (70%) reported less than 70% of the QUADAS items.

Table 1 Study characteristics and assessment of study validity

AUTHORS	YEAR	N=	ILLNESS	AGE	CRITICAL APPRAISAL OF DESCRIPTIONS OF STUDY METHODOLOGY[A]							
					Clear inclusion	Clear conventional test	Clear PCR	Blind index	Blind PCR	Inadequate reported	Withdrawals explained	QUADAS score (%)[b]
Chan et al[29]	2008	196	ARI	0-18 y	No	Yes	Yes	Unclear	Unclear	No	No	64
Roh et al[34]	2008	45	ARI	0-18 y	No	Yes	Yes	Unclear	Unclear	No	No	64
Chiu et al[24]	2008	138	Respiratory symptoms	0-18 y	No	Yes	Yes	Unclear	Yes	No	No	71
Cicek et al[35]	2007	88	LRTI	Unclear	No	Yes	Yes	Unclear	Unclear	No	No	64
Van de Pol et al[36]	2007	181	Respiratory symptoms	Unclear	No	Yes	Yes	Unclear	Unclear	No	No	64
Do Nascimento et al[25]	2007	226	ARI	0-5 y	No	Yes	Yes	Unclear	Unclear	Yes	No	71
Yoo et al[37]	2007	200	ARI	0-5 y	Yes	Yes	Yes	Unclear	Unclear	No	Yes	78
Poehling et al[38]	2006	2797	ARI	0-5 y	Yes	No	Yes	Unclear	Unclear	No	No	64
Kuypers et al[39]	2006	1138	ARI	0-19 y	No	Yes	Yes	Unclear	Yes	Yes	Yes	86
Van de Pol et al[40]	2006	23	LRTI (PICU)	0-5 y	Yes	No	No	Unclear	Unclear	Yes	Yes	71
Rovida et al[41]	2005	135	ARI	Unclear	No	Yes	Yes	Yes	Unclear	Yes	No	79
Alexander et al[42]	2005	193	Respiratory symptoms	Unclear	No	No	No	Unclear	Unclear	Yes	No	57
Garbino et al[43]	2004	11	Respiratory symptoms	Unclear	Yes	No	Yes	Yes	Yes	No	Yes	86

Jennings et al[44]	2004	75	ARI	Unclear	Yes	Yes	No	Unclear	Unclear	No	No	64
Kuypers et al[45]	2004	548	Respiratory symptoms	Unclear	No	Yes	Yes	Unclear	Yes	No	No	71
Vabret et al[27]	2004	362	ARI	Unclear	No	No	No	Unclear	Unclear	No	No	50
Weinberg et al[46]	2004	668	ARI	0-5 y	Yes	No	Yes	Unclear	Unclear	No	No	64
Syrmis et al[47]	2004	598	ARI	0-15 y	No	Yes	Yes	Unclear	Unclear	No	No	64
Erdman et al[48]	2003	470	ARI	0-5 y	No	No	Yes	Unclear	Unclear	No	No	57
Rajala et al[49]	2003	200	LRTI	7 mo-5 y	Yes	Yes	No	Unclear	Unclear	No	No	64
Gueudin et al[50]	2003	75	ARI	Unclear	No	No	Yes	Unclear	Unclear	No	No	57
Poehling et al[51]	2002	233	Respiratory symptoms	0-18 y	Yes	No	Yes	Unclear	Unclear	No	Yes	71
Avellón et al[52]	2001	40	LRTI	Unclear	No	Yes	Yes	Unclear	Unclear	Yes	No	71
Fan et al[53]	1998	69	LRTI	Unclear	No	Yes	No	Unclear	Unclear	No	No	57
Eugene-Ruellan et al[54]	1998	261	Bronchio-litis	Unclear	No	No	Yes	Unclear	Unclear	No	No	57
Freymuth et al[55]	1997	277	URTI/LRTI	0-2 y	No	Yes	Yes	Unclear	Unclear	No	No	64
Valdivia et al[56]	1997	20	Bronchio-litis	Unclear	No	Yes	Yes	Unclear	Unclear	No	No	64
Fan et al[57]	1996	49	LRTI	0-5 y	No	Yes	Yes	Unclear	Unclear	No	No	64
Freymuth et al[26]	1995	238	ARI	0-1 y	No	Yes	Yes	Unclear	Unclear	No	No	64
Yoshio et al[28]	1996	14	Suspec-ted RSV disease	<1 mo	No	No	Yes	Unclear	Unclear	No	No	57
Van Milaan et al[58]	1994	93	URTI/LRTI	Unclear	No	Yes	Yes	Unclear	Unclear	No	No	64
Claas et al[59]	1993	434	Respiratory symptoms	Unclear	No	Yes	Yes	Unclear	Unclear	No	No	64
Paton et al[60]	1992	202	URTI/LRTI	1 mo-6 y	No	No	Yes	Unclear	Unclear	No	No	57

LRTI, lower respiratory tract infection; PICU, pediatric intensive care unit; y, year; ARI, acute respiratory infection; mo, months; URTI, upper respiratory tract infection.

[a] Study validity was assessed scoring relevant items from the Quality Assessment of Diagnostic Accuracy Studies (QUADAS) checklist as Yes, No or Unclear (see Method section).

[b] In total 23 out of 33 (70%) studies reported <70% of QUADAS items

Patients

All studies used different descriptions and/or definitions of respiratory illnesses, i.e. some studies only mentioned the "presence of respiratory symptoms", other studies stated that patients were hospitalized with "acute respiratory infection" or "a combination of upper and lower respiratory infection". Eight studies (24%) included specifically patients with lower respiratory tract infection (including bronchiolitis). In 12 (36%) studies all children were under five years of age. Notably, only 16 (50%) studies reported the actual age (i.e. median age) of the included patient population.

Viral diagnostics methods

All studies that were found all used PCR techniques (i.e. none of the studies used NASBA). Nine studies (23%) used conventional gel detection, 17 studies (44%) used hybridization assays, 5 (13%) nested PCR, and 8 (21%) used real-time PCR.

Table 2 provides detailed characteristics of the viral tests that were used in the different studies. Of the 23 studies evaluating IF, direct and indirect assays were used in 16 (70%) and 5 (22%) studies, respectively. The most commonly used antigen detection kits were from Dako [Denmark, USA, UK or France] and Chemicon [USA], used in 11 (48%) and 6 (26%) studies, respectively. The 28 studies evaluating viral culture used a variety of different cell types. Seventeen (61%) viral culture studies used blind staining with IF or hemadsorption. Studies used a wide variety of target genes in their PCR assays.

Table 2 Viral test characteristics

AUTHORS	YEAR	IF		VIRAL CULTURE		PCR	
		ANTIBODIES USED	TYPE	TYPE OF CELLS	BLIND STAINING	TYPE OF PCR	TARGET GENES
Chan et al[29]	2008	Imagen [Dako, Denmark]	Direct	LLC-MK2, MDCK, RD, HEp-2C	Unclear	Real-time	RSV A/B: L; IV A: M; IV B: NS8
Roh et al[34]	2008			A549, Mv1Lu	Yes	Gel	Seeplex RV [SeeGene, Korea]
Chiu et al[24]	2008	Light Diagnostics [Chemicon, USA]	Direct			I: Gel (RSV) and real-time PCR (IV) II: Hybridisation	I: RSV: F; IV A: M II: microarray
Cicek et al[35]	2007	Respiratory Virus Panel [Bartels, USA]	Indirect	HEp-2, MDCK, AGMK	Yes	Hybridisation	ProDect BCS RV CHIP [bsc Biotech, Italy]
Van de Pol et al[36]	2007	Imagen [Dako, Denmark]	Direct	LCC-MK2, RD, R-HeLa and HEp-2C	No	Real-time	RSV A/B: N; IV A: M; IV B: HA; PIV 1-3: HN; PIV 4: N; AdV: Hexon
Do Nascimento et al[25]	2007	Respiratory Virus Panel [Biotrin, Ireland]	Indirect			I: Gel II: Nested	I: RSV: F/G II: RSV: F/G
Yoo et al[37]	2007	Imagen [Dako, Denmark]	Direct			Gel	Seeplex RV [SeeGene, Korea]
Poehling et al[38]	2006			Primary rhesus monkey kidney , HEp-2C and NCI-H292	Yes	Hybridisation	Unclear
Kuypers et al[39]	2006	[Chemicon, USA]	Indirect			Real-time	RSV: M; IV A: M; PIV 1-2: L; PIV 3: M; AdV: Hexon
Van de Pol et al[40]	2006	Imagen [Dako, Denmark]	Direct	LCC-MK2, RD, R-HeLa and HEp-2C	No	Real-time	RSV: N; IV A: M; IV B: HA; PIV 1-4: Unclear; AdV: Unclear
Rovida et al[41]	2005	SimulFluor [Chemicon, USA]	Direct	A549, Mv1Lu, HEp-2, LLC-MK2 and MDCK	Yes	Gel	RSV: F; IV A/B: HA; PIV 1-3: HN; AdV: Hexon
Alexander et al[42]	2005	Directigen [Dickenson, USA]	Direct	Unclear	Yes	Gel	IV A: M; IV B: NS1
Garbino et al[43]	2004			Human embryonic fibroblasts, A549, MDCK, and LLC-MK2	Unclear	Real-time PCR	RSV: N; IVA M; IVB: HA; PIV 1+3 HN
Jennings et al[44]	2004	Imagen [Dako, USA]	Direct	HEL, LLC-MK2, MDCK, HEp-2C and A549	Yes	Gel	RSV: unclear, IV A/B M, PIV 1-3: HN
Kuypers et al[45]	2004	[Chemicon, USA]	Indirect			Real-time	RSV A/B: M
Vabret et al I+II[27]	2004	Imagen [Dako, UK]	Direct	NCI-H292 and MRC-5	Yes	Hybridisation	I AdV: VA II AdV: VA

Weinberg et al[46]	2004			Primary rhesus monkey kidney , HEp-2C and NCI-H292	Yes	Hybridisation	Unclear
Syrmis et al[47]	2004	Bartel's [Intracel, USA]	Direct	MRC-5, A549, HEp-2C and LLC-MK2	Yes	Hybridisation	RSV: L, IV A/B: NS , PIV 1: HN, PIV 2-3 N, AdV: Hexon
Erdman et al[48]	2003	reagents from [Argene biosoft, France]	Direct	HEp-2C and NCI-H292	Yes	Hybridisation	RSV: F; PIV 1-3 HN; IV A/B: NS1
Rajala et al[49]	2003			HEp-2C	Yes	Nested	RSV: part of F and G
Gueudin et al[50]	2003	Imagen [Dako, France]	Direct	MRC-5 and A549	Yes	Real-time	RSV: N
Poehling et al[51]	2002			rhesus monkey kidney	Yes	Hybridisation	IV: M
Avellón et al[52]	2001	[Chemicon, USA]	Unclear	A549 and human lung fibroblasts	No	Nested	AdV: Hexon
Fan et al[53]	1998			CMK and LLC-MK2	Yes	Hybridisation	RSV A/B: 1B and N; IV A: M; IV B: NS1; PIV 1-3: HN
Eugene-Ruellan et al[54]	1998	[Dako, UK]	Direct	MRC-5 and NCI-H292	No	Hybridisation	RSV A/B and PIV 3: L
Freymuth et al[55]	1997	[Dako, UK]	Unclear	MRC-5 and NCI-H292	Yes	Hybridisation	RSV: N, PIV 3: HN; AdV: Hexon
Valdivia et al[56]	1997	provided by collegue	Indirect	HEp-2C	No	Nested	RSV: N
Fan et al[57]	1996			CMK, LLC-MK2, AGMK, HEp-2C, MDCK and HFS	Yes	Hybridisation	PIV 1: HN
Freymuth et al I+II[26]	1995	Imagen [Dako, UK]	Direct	MRC-5 and NCI-H292	No	Hybridisation	I RSV: N II RSV: 1B
Yoshio et al I+II[28]	1996			HEp-2C	No	1 Gel II Nested	RSV: F
Van Milaan et al[58]	1994	[Chemicon, USA]	Direct	HEp-2C	No	Hybridisation	RSV: starts at 1B and ends in N
Claas et al[59]	1993	Imagen [Dako, Denmark]	Direct	Tertiary rhesus monkey kidney	Yes	Hybridisation	IV A/B: NS
Paton et al[60]	1992			HEp-2C and A549	Unclear	Gel	RSV A/B: F

Cells from: *LLC-MK2, Monkey Rhesus kidney; RD, human rhabdomyosarcoma; R-HeLa, rhinovirus-sensitive human cervical cancer; HEp-2C, human larynx carcinoma; A549, human lung carcinoma; Mv1Lu, mustela vison (mink) fetal lung; MDCK, Madin-Darby canine kidney; HEL, human embryonic lung; NCI-H292, mucoepidermoid carcinoma from human lungs; MRC-5, human fetal lung; CMK, human megakaryoblastic; AGMK, African Green Monkey Kidney.*

Genes: *N, nucleocapsid; M, matrix gene; HA, hemagglutinin; NS, nonstrucural; HN, Hemagglutinin-neuraminidase; F, gene encoding the F-protein; L, polymerase gene; VA, virus-associated.*

[a] *Described in the Nation Center for Biotechnology Information GEO platform GPL2429.*

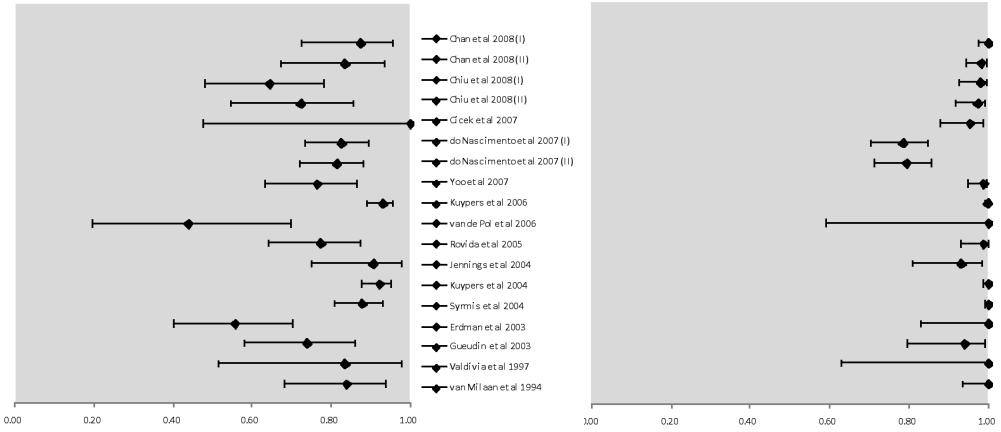
Diagnostic accuracy of conventional tests versus PCR

Immunofluorescence

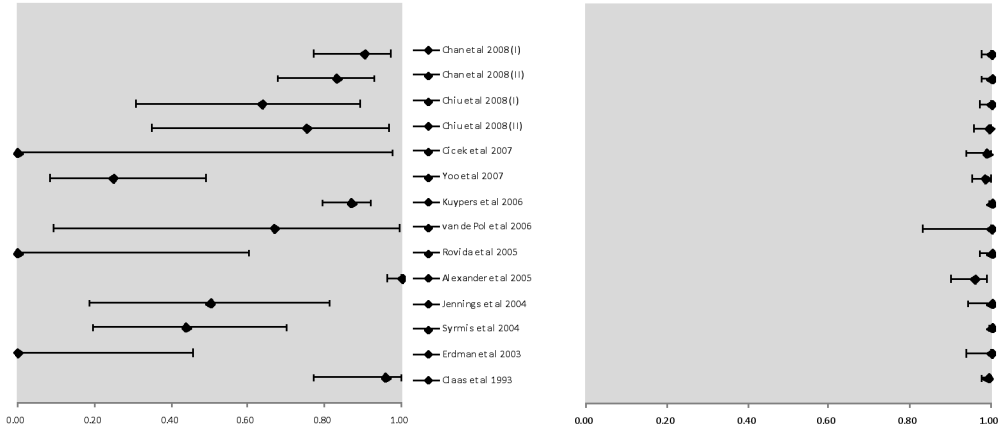
Eighteen, 14, 9, and 9 studies reported IF results for RSV, IV, PIV, and AdV, respectively. Figures 2a-d show the sensitivity and specificity of immunofluorescence for each of these viruses. Heterogeneity could not be excluded with I2 ranging from 0.69 to 0.90, i.e. pooling of results was not possible. For RSV the sensitivity ranged from 0.44 to 1.00. The sensitivity for IV, PIV, and AdV ranged from 0.00 to 1.00. Specificity ranged from 0.78 to 1.00.

Figure 2

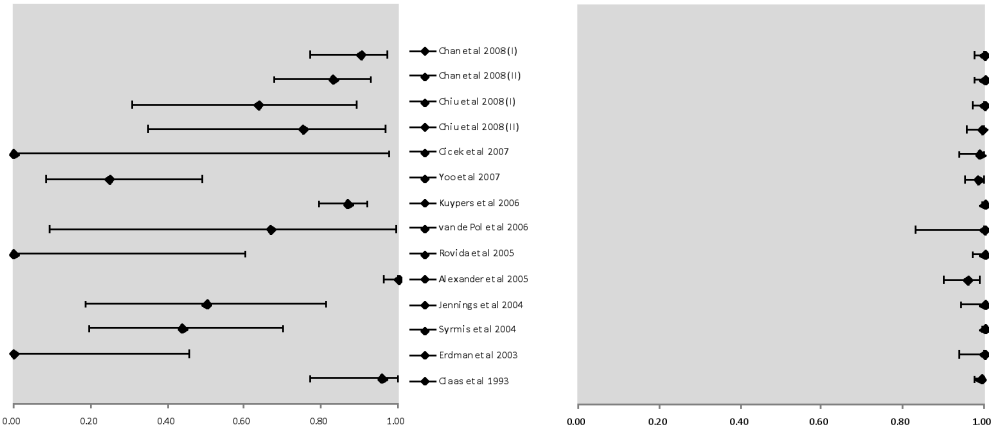
(A) RSV: Sensitivity and specificity of immunofluorescence for RSV compared to PCR as the reference method



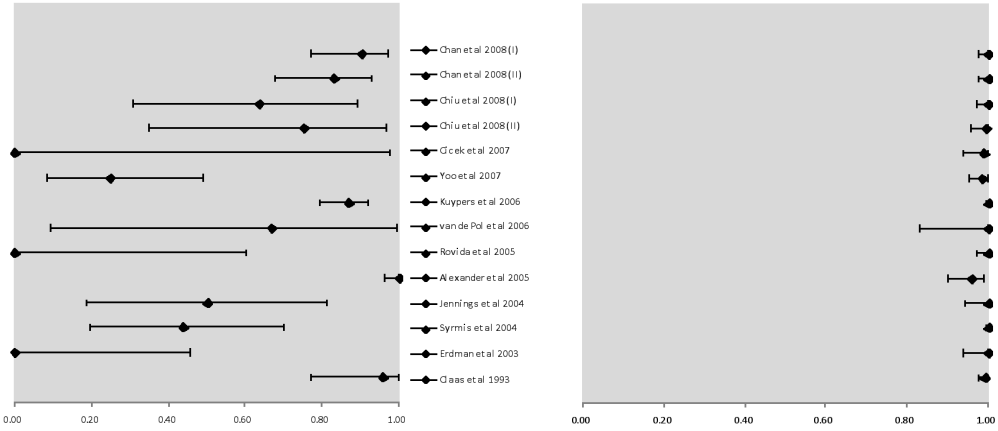
(B) IV: Sensitivity and specificity of immunofluorescence for influenza virus compared to PCR as the reference method



(C) PIV: Sensitivity and specificity of immunofluorescence for parainfluenza viruses compared to PCR as the reference method



(D) AdV: Sensitivity and specificity of immunofluorescence for adenoviruses compared to PCR as the reference method



Diamonds represent point estimates of sensitivity and specificity; lines represent 95% confidence intervals.

Viral culture

Eighteen, 14, 11, and 7 studies reported results on viral cultures for RSV, IV, PIV, and AdV, respectively. Pooling was not possible due to heterogeneity (I2 range: 0.58 to 0.94). The sensitivity estimates of the individual studies ranged from 0.00 to 1.00. Specificities ranged from 0.76 to 1.00. However, most studies (90%) reported a specificity of 0.95 or more.

Combination of immunofluorescence and viral culture

Thirteen RSV studies, 8 IV studies, 7 PIV studies, and 8 AdV studies reported results on both IF and viral culture combined. Heterogeneity was large (I^2>0.69), and the sensitivity of IF and viral culture combined again varied considerably (0.40 to 1.00 for RSV, IV, and PIV; 0.10 to 1.00 for AdV). Specificity estimates of the combination of viral culture and IF ranged from 0.85 to 1.00.

Exploration of heterogeneity

To explore heterogeneity, subgroup analyses were performed for the different respiratory illnesses, age groups, and PCR types. Patient age and type of PCR used did not explain the observed heterogeneity, i.e. heterogeneity was found within these subgroups as well.

Discussion

Principle findings

For the present systematic review, 33 studies were identified that compared the results of conventional diagnostics (IF and viral culture) to NATs -as the reference standard- in hospitalized infants and children with respiratory symptoms. Study results varied considerably. In individual studies the sensitivity of conventional tests ranged from 0.00 to 1.00.

Strengths and weaknesses

The major strengths of our study comprise 1) the extensive search strategy, 2) independent selection, quality assessment, and data-abstraction by two reviewers, and 3) minimizing the problem of incomplete reporting by contacting authors when necessary, and 4) focus on studies reporting clinical information such as patients' symptoms and hospitalization status. Nevertheless, some of our findings deserve further discussion. First, a problem inherent to systematic reviews is that reviews can never be better than the individual studies that are included. Twenty-three of the 33 individual publications (70%) evaluated in our review reported less than 70% of the QUADAS quality checklist. Problems in the study methods included absence of clinical criteria for patient inclusion, lack of blinding of test results, no information on inadequate test results, and no information on withdrawals of patients. Second, publication bias could not be ruled out completely. A funnel plot of the included studies (data not shown) indeed appeared to be asymmetric (smaller studies showing higher sensitivities of conventional

viral tests) indicating possible bias. On the other hand, publication bias could not reliably be assessed due to between-study heterogeneity (30). Third, some relevant data could not be abstracted from publications due to incomplete reporting. Detailed reporting of respiratory illnesses and patient age would have allowed more comprehensive subgroup analyses.

Heterogeneity

Our systematic review clearly shows high between-study heterogeneity for the sensitivity and specificity of conventional tests relative to NATs, which could not be explained by variations in respiratory illness, age, and the types of NATs used. A major reason for the observed heterogeneity may be the extremely large variety of approaches, systems and reagents used for both viral culture and NATs. For viral culture varying numbers of different cell types were used, and some studies used blind staining or hemadsorption whereas others did not. NAT techniques were mostly in house developed and also varied widely in approaches and target genes chosen and reagents and platforms used. Consequently, sensitivity of different NAT assays may differ as much as 1000-fold as has been shown by results from international proficiency testing studies (31;32). Other factors that may have impacted the results are the type of specimens used, transport and processing of samples, and the time point of sample submission in the context of clinical illness. It seems clear that meaningful global comparisons of different test approaches are currently impossible due to large variation between studies in a single NAT category (i.e. within the subgroup of studies using PCR with gel detection or within the subgroup of studies using real-time PCR). Unfortunately, International Standards for respiratory viruses are lacking. The only possibility for laboratories to compare their performance with others is offered by external quality assessment schemes, for instance those organized by Quality Control for Molecular Diagnostics (QCMD; www.qcmd.org). Performance of viral tests depends largely on careful optimization and meticulous execution of laboratory protocols (33).

Implications

In the present systematic review an enormous variation was found in the sensitivity of conventional viral tests compared to NATs as the reference method, for the detection of viruses in hospitalized infants and children with respiratory illness. This underscores the need for improved standardization of diagnostic methods for diagnosis of viral respiratory infections. International standards are needed to improve viral diagnosis and patient management, including adequate (future) use of antiviral drugs, and optimal infection control.

Acknowledgements

The study was supported by an “Alexandre Suerman program” MD/PhD grant from the University Medical Center Utrecht, The Netherlands.

Reference List

(1) Henrickson KJ, Hoover S, Kehl KS, Hua W. National disease burden of respiratory viruses detected in children by polymerase chain reaction. Pediatr Infect Dis J 2004;23:S11-S18.
(2) Shay DK, Holman RC, Newman RD, Liu LL, Stout JW, Anderson LJ. Bronchiolitis-associated hospitalizations among US children, 1980-1996. JAMA 1999;282:1440-6.
(3) Weigl JA, Puppe W, Grondahl B, Schmitt HJ. Epidemiological investigation of nine respiratory pathogens in hospitalized children in Germany using multiplex reverse-transcriptase polymerase chain reaction. Eur J Clin Microbiol Infect Dis 2000;19:336-43.
(4) Yanney M, Vyas H. The treatment of bronchiolitis. Arch Dis Child 2008;93:793-8.
(5) Harper SA, Bradley JS, Englund JA, File TM, Gravenstein S, Hayden FG, et al. Seasonal influenza in adults and children--diagnosis, treatment, chemoprophylaxis, and institutional outbreak management: clinical practice guidelines of the Infectious Diseases Society of America. Clin Infect Dis 2009;48:1003-32.
(6) Henrickson KJ, Hall CB. Diagnostic assays for respiratory syncytial virus disease. Pediatr Infect Dis J 2007;26:S36-S40.
(7) Ieven M. Currently used nucleic acid amplification tests for the detection of viruses and atypicals in acute respiratory infections. J Clin Virol 2007;40:259-76.
(8) Macfarlane P, Denham J, Assous J, Hughes C. RSV testing in bronchiolitis: which nasal sampling method is best? Arch Dis Child 2005;90:634-5.
(9) Bellau-Pujol S, Vabret A, Legrand L, Dina J, Gouarin S, Petitjean-Lecherbonnier J, et al. Development of three multiplex RT-PCR assays for the detection of 12 respiratory RNA viruses. J Virol Methods 2005;126:53-63.
(10) Gilbert LL, Dakhama A, Bone BM, Thomas EE, Hegele RG. Diagnosis of viral respiratory tract infections in children by using a reverse transcription-PCR panel. J Clin Microbiol 1996;34:140-3.
(11) Kehl SC, Henrickson KJ, Hua W, Fan J. Evaluation of the Hexaplex assay for detection of respiratory viruses in children. J Clin Microbiol 2001;39:1696-701.
(12) Akobeng AK. Understanding systematic reviews and meta-analysis. Arch Dis Child 2005;90:845-8.

(13) Whiting P, Rutjes AW, Reitsma JB, Bossuyt PM, Kleijnen J. The development of QUADAS: a tool for the quality assessment of studies of diagnostic accuracy included in systematic reviews. BMC Med Res Methodol 2003;3:25.
(14) Whiting PF, Weswood ME, Rutjes AW, Reitsma JB, Bossuyt PN, Kleijnen J. Evaluation of QUADAS, a tool for the quality assessment of diagnostic accuracy studies. BMC Med Res Methodol 2006;6:9.
(15) Zamora J, Abraira V, Muriel A, Khan K, Coomarasamy A. Meta-DiSc: a software for meta-analysis of test accuracy data. BMC Med Res Methodol 2006;6:31.
(16) Higgins JP, Thompson SG, Deeks JJ, Altman DG. Measuring inconsistency in meta-analyses. BMJ 2003;327:557-60.
(17) Cochrane Handbook for Systematic Reviews of Interventions 4.2.5. Available at: http://www.cochrane.dk/cochrane/handbook/hbook.htm. Accessed November 30, 2006
(18) Agoritsas K, Mack K, Bonsu BK, Goodman D, Salamon D, Marcon MJ. Evaluation of the Quidel QuickVue test for detection of influenza A and B viruses in the pediatric emergency medicine setting by use of three specimen collection methods. J Clin Microbiol 2006;44:2638-41.
(19) Aguilar JC, Perez-Brena MP, Garcia ML, Cruz N, Erdman DD, Echevarria JE. Detection and identification of human parainfluenza viruses 1, 2, 3, and 4 in clinical samples of pediatric patients by multiplex reverse transcription-PCR. J Clin Microbiol 2000;38:1191-5.
(20) Freymuth F, Vabret A, Cuvillon-Nimal D, Simon S, Dina J, Legrand L, et al. Comparison of multiplex PCR assays and conventional techniques for the diagnostic of respiratory virus infections in children admitted to hospital with an acute respiratory illness. J Med Virol 2006;78:1498-504.
(21) Hibbitts S, Rahman A, John R, Westmoreland D, Fox JD. Development and evaluation of NucliSens basic kit NASBA for diagnosis of parainfluenza virus infection with 'end-point' and 'real-time' detection. J Virol Methods 2003;108:145-55.
(22) Laundy M, jayi-Obe E, Hawrami K, Aitken C, Breuer J, Booy R. Influenza A community-acquired pneumonia in East London infants and young children. Pediatr Infect Dis J 2003;22:S223-S227.
(23) Sung RY, Chan PK, Choi KC, Yeung AC, Li AM, Tang JW, et al. Comparative study of nasopharyngeal aspirate and nasal swab specimens for diagnosis of acute viral respiratory infection. J Clin Microbiol 2008;46:3073-6.
(24) Chiu CY, Urisman A, Greenhow TL, Rouskin S, Yagi S, Schnurr D, et al. Utility of DNA microarrays for detection of viruses in acute respiratory tract infections in children. J Pediatr 2008;153:76-83.
(25) do Nascimento CA, Leal AL, Souza TS, de Moraes CT, Comone P, Tenorio EC, et al. One-step reverse transcriptase polymerase chain reaction for the diagnosis of respiratory syncytial virus in children. J Virol Methods 2008;148:115-9.

(26) Freymuth F, Eugene G, Vabret A, Petitjean J, Gennetay E, Brouard J, et al. Detection of respiratory syncytial virus by reverse transcription-PCR and hybridization with a DNA enzyme immunoassay. J Clin Microbiol 1995;33:3352-5.
(27) Vabret A, Gouarin S, Joannes M, Barranger C, Petitjean J, Corbet S, et al. Development of a PCR-and hybridization-based assay (PCR Adenovirus Consensus) for the detection and the species identification of adenoviruses in respiratory specimens. J Clin Virol 2004;31:116-22.
(28) Yoshio H, Yamada M, Nii S. Reverse transcription-polymerase chain reaction amplification of respiratory syncytial virus genome from neonatal nasal swab samples. Acta Paediatr Jpn 1996;38:429-33.
(29) Chan KH, Peiris JS, Lim W, Nicholls JM, Chiu SS. Comparison of nasopharyngeal flocked swabs and aspirates for rapid diagnosis of respiratory viruses in children. J Clin Virol 2008;42:65-9.
(30) Lau J, Ioannidis JP, Terrin N, Schmid CH, Olkin I. The case of the misleading funnel plot. BMJ 2006;333:597-600.
(31) Van Vliet KE, Muir P, Echevarria JM, Klapper PE, Cleator GM, van Loon AM. Multicenter proficiency testing of nucleic acid amplification methods for the detection of enteroviruses. J Clin Microbiol 2001;39:3390-2.
(32) Templeton KE, Forde CB, Loon AM, Claas EC, Niesters HG, Wallace P, et al. A multi-centre pilot proficiency programme to assess the quality of molecular detection of respiratory viruses. J Clin Virol 2006;35:51-8.
(33) Niesters HG, Puchhammer-Stockl E. Standardisation and controls, why can't we overcome the hurdles? J Clin Virol 2004 October;31(2):81-3.
(34) Roh KH, Kim J, Nam MH, Yoon S, Lee CK, Lee K, et al. Comparison of the Seeplex reverse transcription PCR assay with the R-mix viral culture and immunofluorescence techniques for detection of eight respiratory viruses. Ann Clin Lab Sci 2008;38:41-6.
(35) Cicek C, Gulen F, Karatas E, Demir E, Tanac R, Bacakoglu F, et al. Comparison of the "ProDect BCS RV CHIP" assay with the combination of shell vial cell culture and immunofluorescence antibody test for the detection of respiratory viruses. J Virol Methods 2007;143:161-8.
(36) van de Pol AC, van Loon AM, Wolfs TF, Jansen NJ, Nijhuis M, Breteler EK, et al. Increased detection of respiratory syncytial virus, influenza viruses, parainfluenza viruses, and adenoviruses with real-time PCR in samples from patients with respiratory symptoms. J Clin Microbiol 2007;45:2260-2.
(37) Yoo SJ, Kuak EY, Shin BM. Detection of 12 respiratory viruses with two-set multiplex reverse transcriptase-PCR assay using a dual priming oligonucleotide system. Korean J Lab Med 2007;27:420-7.
(38) Poehling KA, Edwards KM, Weinberg GA, Szilagyi P, Staat MA, Iwane MK, et al. The underrecognized burden of influenza in young children. N Engl J Med 2006 ;355:31-40.

(39) Kuypers J, Wright N, Ferrenberg J, Huang ML, Cent A, Corey L, et al. Comparison of real-time PCR assays with fluorescent-antibody assays for diagnosis of respiratory virus infections in children. J Clin Microbiol 2006;44:2382-8.
(40) van de Pol AC, Wolfs TF, Jansen NJ, van Loon AM, Rossen JW. Diagnostic value of real-time polymerase chain reaction to detect viruses in young children admitted to the paediatric intensive care unit with lower respiratory tract infection. Crit Care 2006;10:R61.
(41) Rovida F, Percivalle E, Zavattoni M, Torsellini M, Sarasini A, Campanini G, et al. Monoclonal antibodies versus reverse transcription-PCR for detection of respiratory viruses in a patient population with respiratory tract infections admitted to hospital. J Med Virol 2005;75:336-47.
(42) Alexander R, Hurt AC, Lamb D, Wong FY, Hampson AW, Barr IG. A comparison of a rapid test for influenza with laboratory-based diagnosis in a paediatric population. Commun Dis Intell 2005;29:272-6.
(43) Garbino J, Gerbase MW, Wunderli W, Deffernez C, Thomas Y, Rochat T, et al. Lower respiratory viral illnesses: improved diagnosis by molecular methods and clinical impact. Am J Respir Crit Care Med 2004;170:1197-203.
(44) Jennings LC, Anderson TP, Werno AM, Beynon KA, Murdoch DR. Viral etiology of acute respiratory tract infections in children presenting to hospital: role of polymerase chain reaction and demonstration of multiple infections. Pediatr Infect Dis J 2004;23:1003-7.
(45) Kuypers J, Wright N, Morrow R. Evaluation of quantitative and type-specific real-time RT-PCR assays for detection of respiratory syncytial virus in respiratory specimens from children. J Clin Virol 2004;31:123-9.
(46) Weinberg GA, Erdman DD, Edwards KM, Hall CB, Walker FJ, Griffin MR, Schwartz B. Superiority of reverse-transcription polymerase chain reaction to conventional viral culture in the diagnosis of acute respiratory tract infections in children. J Infect Dis 2004;189:706-10.
(47) Syrmis MW, Whiley DM, Thomas M, Mackay IM, Williamson J, Siebert DJ, et al. A sensitive, specific, and cost-effective multiplex reverse transcriptase-PCR assay for the detection of seven common respiratory viruses in respiratory samples. J Mol Diagn 2004;6:125-31.
(48) Erdman DD, Weinberg GA, Edwards KM, Walker FJ, Anderson BC, Winter J, et al. GeneScan reverse transcription-PCR assay for detection of six common respiratory viruses in young children hospitalized with acute respiratory illness. J Clin Microbiol 2003;41:4298-303.
(49) Rajala MS, Sullender WM, Prasad AK, Dar L, Broor S. Genetic variability among Group A and B respiratory syncytial virus isolates from a large referral hospital in New Delhi, India. J Clin Microbiol 2003;41:2311-6.
(50) Gueudin M, Vabret A, Petitjean J, Gouarin S, Brouard J, Freymuth F. Quantitation of respiratory syncytial virus RNA in nasal aspirates of children by real-time RT-PCR assay. J Virol Methods 2003;109:39-45.

(51) Poehling KA, Griffin MR, Dittus RS, Tang YW, Holland K, Li H, Edwards KM. Bedside diagnosis of influenzavirus infections in hospitalized children. Pediatrics 2002;110:83-8.
(52) Avellon A, Perez P, Aguilar JC, Lejarazu R, Echevarria JE. Rapid and sensitive diagnosis of human adenovirus infections by a generic polymerase chain reaction. J Virol Methods 2001;92:113-20.
(53) Fan J, Henrickson KJ, Savatski LL. Rapid simultaneous diagnosis of infections with respiratory syncytial viruses A and B, influenza viruses A and B, and human parainfluenza virus types 1, 2, and 3 by multiplex quantitative reverse transcription-polymerase chain reaction-enzyme hybridization assay (Hexaplex). Clin Infect Dis 1998;26:1397-402.
(54) Eugene-Ruellan G, Freymuth F, Bahloul C, Badrane H, Vabret A, Tordo N. Detection of respiratory syncytial virus A and B and parainfluenzavirus 3 sequences in respiratory tracts of infants by a single PCR with primers targeted to the L-polymerase gene and differential hybridization. J Clin Microbiol 1998;36:796-801.
(55) Freymuth F, Vabret A, Galateau-Salle F, Ferey J, Eugene G, Petitjean J, et al. Detection of respiratory syncytial virus, parainfluenzavirus 3, adenovirus and rhinovirus sequences in respiratory tract of infants by polymerase chain reaction and hybridization. Clin Diagn Virol 1997;8:31-40.
(56) Valdivia A, Savon C, Chacon D, Sarmiento L, Morier L, Otero A, et al. Analysis of respiratory syncytial virus in clinical samples by reverse transcriptase-polymerase chain reaction restriction mapping. Mem Inst Oswaldo Cruz 1997;92:389-93.
(57) Fan J, Henrickson KJ. Rapid diagnosis of human parainfluenza virus type 1 infection by quantitative reverse transcription-PCR-enzyme hybridization assay. J Clin Microbiol 1996;34:1914-7.
(58) van Milaan AJ, Sprenger MJ, Rothbarth PH, Brandenburg AH, Masurel N, Claas EC. Detection of respiratory syncytial virus by RNA-polymerase chain reaction and differentiation of subgroups with oligonucleotide probes. J Med Virol 1994;44:80-7.
(59) Claas EC, van Milaan AJ, Sprenger MJ, Ruiten-Stuiver M, Arron GI, Rothbarth PH, Masurel N. Prospective application of reverse transcriptase polymerase chain reaction for diagnosing influenza infections in respiratory samples from a children's hospital. J Clin Microbiol 1993;31:2218-21.
(60) Paton AW, Paton JC, Lawrence AJ, Goldwater PN, Harris RJ. Rapid detection of respiratory syncytial virus in nasopharyngeal aspirates by reverse transcription and polymerase chain reaction amplification. J Clin Microbiol 1992;30:901-4.

CHAPTER 5

Human bocavirus and KI/WU polyomaviruses in pediatric intensive care patients

Alma C. van de Pol
Tom F.W. Wolfs
Nicolaas J.G. Jansen
Jan L.L. Kimpen
Anton M. van Loon
John W.A. Rossen

Emerg Infect Dis 2009;15:454-7

Abstract

We evaluated the prevalence of human bocavirus and KI and WU polyomaviruses in pediatric intensive care patients with and without lower respiratory tract infection (LRTI). The prevalence of these viruses was 5.1%, 0%, and 2.6%, respectively, in children with LRTI and 4.8%, 4.8%, and 2.4%, respectively, in those without LRTI.

Introduction

Through use of molecular diagnostic tests such as real-time PCR in the clinical setting, our scope of etiologic viral agents of lower respiratory tract infection (LRTI) has increased. Respiratory viruses can now be detected in most pediatric intensive care patients with LRTI (1). Recently, 3 new viruses were described: human bocavirus (hBoV) and KI (KIPyV) and WU (WUPyV) polyomaviruses (2-4). These viruses were first identified in respiratory samples obtained from children with respiratory tract infections. An association between the viruses and respiratory tract symptoms was postulated, but, to date, evidence supporting that association is incomplete (5-7). This study evaluates the prevalences of hBoV, KIPyV, and WUPyV infections in pediatric intensive care patients with acute respiratory insufficiency caused by LRTI.

Methods

Patients <5 years of age who were admitted for LRTI to the pediatric intensive care unit (PICU) of Wilhelmina Children's Hospital, Utrecht, the Netherlands, were enrolled from October through May during 2005–2008. Patients were excluded if they had any of the following: asthma exacerbation, immunocompromised state, indication for antimicrobial drugs other than for LRTI, and repeated PICU admission for LRTI during the study period. Control group participants were children <18 years of age (median 2.2 years) who were admitted to the PICU from October 2005 through March 2006 for reasons other than LRTI.

Clinical data were obtained by using standardized forms to extract data from electronic charts. Underlying illnesses were defined as chronic pulmonary disease, congenital heart disease, immunodeficiency, malignancy, neurologic disease, or gastrointestinal disease (8).

To assess the severity of illness, we used the lowest ratio during the first 24 hours of the partial pressure of oxygen in arterial blood (PaO_2) to the inspired oxygen fraction (FiO_2). These ratios were acquired from the Pediatric Intensive Care Evaluation database, which contains validated clinical data for all Dutch PICU admissions.

Nasopharyngeal aspirates were collected from all patients in the LRTI group as part of the investigation of their illnesses. In the control group, nasopharyngeal aspirates were taken from intubated patients, and throat swabs were taken from extubated children as part of routine surveillance to identify transmission of respiratory syncytial virus (RSV). Because RSV surveillance was conducted as part of normal patient care, patient consent/ethical approval was not needed, according to the Medical Ethical Research Council of our institution.

Specimens from patients in the LRTI group were initially examined for RSV, influenza viruses, parainfluenza viruses, adenoviruses, rhinoviruses, coronaviruses, human metapneumovirus, and Mycoplasma pneumoniae by using real-time PCR as previously described (1;9;10). Specimens from patients in the control group were initially examined for RSV. All samples were retrospectively tested for hBoV, KIPyV, and WUPyV also by using real-time PCR as previously described (11;12). After nucleic acid extraction using the MagNA Pure LC 1.0 nucleic acid isolation system (Roche Diagnostics, Rotkreuz, Switzerland), amplification was carried out in a 25-µL reaction mixture on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Positive controls for the KIPyV and WUPyV PCR were provided by S. Bialasiewicz and T.P. Sloots, University of Queensland, Queensland, Australia, and the positive control for hBoV was provided by T. Allander, Karolinska Institute, Stockholm, Sweden. Internal control viruses were used to monitor efficient extraction and amplification. Real-time PCR results were expressed in cycle threshold (Ct) values. Ct values are inversely correlated with viral load; i.e., low Ct values indicate high viral loads.

Results

Of 90 LRTI patients enrolled, 78 (86.7%) had sufficient material stored for hBoV, KIPyV, and WUPyV testing. Eighty-eight control patients were enrolled, of which 83 (94.3%) had sufficient material stored to be included. Table 1 provides patients' demographic and clinical characteristics. The main clinical conditions of control patients were cardiac disease requiring surgery (33.7%), trauma (8.4%), sepsis (8.4%), and upper respiratory tract infection (8.4%). A total of 57 (68.7%) nasopharyngeal aspirates and 26 (31.3%) throat swabs from the 83 patients who had sufficient samples were tested.

In LRTI patients, hBoV was found in 4 (5.1%) and WUPyV in 2 (2.6%) of the 78 patients. No samples tested positive for KIPyV. Table 2 shows Ct values and clinical characteristics for LRTI patients whose samples were positive as well as for controls whose samples were positive for these viruses. Other respiratory viruses were found in 70 (89.7%) of the 78 children. RSV was found in 52 (66.7%), influenza viruses in 3 (3.8%), parainfluenza viruses in 2 (2.6%), adenoviruses in 4 (5.1%), rhinoviruses in 20 (25.6%), coronaviruses in 6 (7.7%), human metapneumovirus in 5 (6.4%), and *Mycoplasma pneumoniae* in 1 (1.3%) of the patients. Multiple respiratory viruses were found in 3 of the 4 LRTI patients with hBoV infection and in both patients with WuPyV infection (Table 2). One patient had a single infection with hBoV (i.e., no other virus was detected). This patient was born at 31 weeks of gestational age and had a history of a grade IV idiopathic respiratory distress syndrome. She was admitted to the PICU at 19 months of age with a severe LRTI. Bacterial throat and blood cultures remained negative.

In the control group, hBoV was found in samples from 4 (4.8%) patients, KIPyV was found in 4 (4.8%), and WUPyV was present in 2 (2.4%) samples from the 83 patients whose samples could be tested. One patient was found to have a co-infection with hBoV and KIPyV.

Median Ct values for hBoV, KIPyV, and WUPyV combined were 18.1 (interquartile range [IQR] 20.4) for the LRTI group and 34.4 (IQR 5.1) for the control group ($p = 0.09$; Figure 1). The Ct values indicate that, on average, the viral load in the LRTI group might be higher than in the control group.

Table 1 Demographic and clinical characteristics for PICU patients with LTRIs and for controls

CHARACTERISTICS OF PATIENTS	LRTI GROUP, N = 78	CONTROL GROUP, N = 83
Male, no. (%)	43 (55.1)	44 (53.0)
Nasopharyngeal aspirates, no. (%)	78 (100.0)	57 (68.7)
Mechanical ventilation, no. (%)	76 (97.4)	79 (95.2)
Age, mo, median (IQR)	1.5 (4.2)	26.1 (134)
PaO2/FiO2, mm Hg, median (IQR)	130 (74.3)	250 (206)
Time on ventilator, d, median (IQR)	9 (5)	4 (9)
Time in hospital, d, median (IQR)	10 (6)	6 (11)

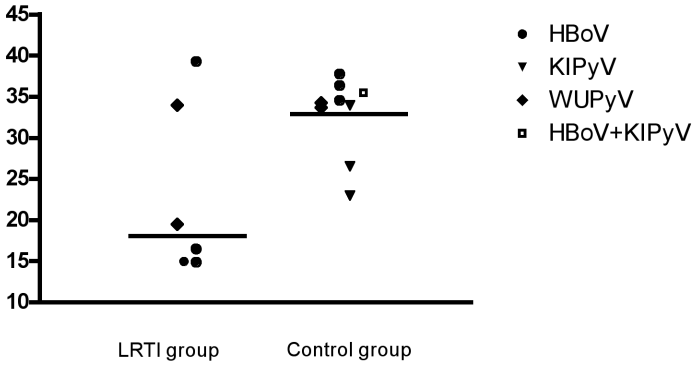
LRTI, lower respiratory tract infection; IQR, interquartile range; PaO2/FiO2, ratio of the partial pressure of oxygen in arterial blood to the inspired oxygen fraction.

Table 2 Clinical characteristics of patients with HBoV, KIPyV, and WUPyV infection

	ADMISSION DIAGNOSIS	VIRUS (CT VALUE)	SAMPLE TYPE	SEX (M/F)	AGE (MONTH)	IMMUNO COMPROMISED	OTHER UNDERLYING DISEASE	LENGTH OF STAY (DAYS)	OTHER VIRUSES
LRTI group									
1	LRTI	HBoV (39.4)	NPA	F	13	No	Pulmonary dysplasia (home mechanical ventilation)	10	Adeno hMPV
2	LRTI	HBoV (15.0)	NPA	F	9	No	IRDS, PVL	10	Adeno
3	LRTI	HBoV (16.6)	NPA	M	13	No	Recurrent wheezing	5	RSV
4	LRTI	HBoV (15.0)	NPA	F	19	No	IRDS	7	None
5	LRTI	WUPyV (19.6)	NPA	M	1	No	None	11	RSV
6	LRTI	WUPyV (34.1)	NPA	F	41	No	Mitochondrial encephalopathy	18	Influenzavirus
Control group									
7	Sepsis	HBoV (36.5)	NPA	F	27	No	None	7	NA
8	Infectious meningitis	HBoV (34.7)	NPA	F	100	No	22Q11 deletion (cardiac and neurological disease)	3	NA
9	URTI	HBoV (37.9)	NPA	M	41	No	Spinal muscular atrophy	74	NA
10	Observation after brain biopsy	HBoV/ KIPyV (32/39)	NPA	F	127	Yes	AML, BMT, Brochiolitis obliterans	46	NA
11	VSD closure	WUPyV (33.8)	Swab	F	15	No	VSD	2	NA
12	Septic shock	WUPyV (34.4)	NPA	M	40	No	None	19	NA
13	Cardiac malformation	KIPyV (34.1)	NPA	F	4	No	Cardiac malformation	14	NA
14	Septic shock	KIPyV (23.1)	NPA	M	152	Yes	AML, Aplasia	11	NA
15	ALTE	KIPyV (26.7)	NPA	M	2	No	None	8	NA

LRTI, lower respiratory tract infection; HBoV, human bocavirus; KIPyV, KI polyomavirus; WUPyV, WU polyomavirus; Ct, cycle threshold; NPA, nasopharyngeal aspirate; Adeno, adenovirus; hMPV, human metapneumovirus; RSV, respiratory syncytial virus; IRDS, idiopathic respiratory distress syndrome; PVL, periventricular leukomalacia; NA, not assessed; URTI, upper respiratory tract infection; AML, acute myeloid leukemia; BMT, bone marrow transplant; VSD, ventricular septal defect; ALTE, apparent life-threatening event.

Figure 1 Ct values of LRTI and control patients with hBoV, KIPyV, and (WUPyV) infections



Horizontal bars represent group medians (difference 16.3 Ct, p = 0.09). Ct, cycle threshold; LRTI, lower respiratory tract infection; hBoV, human bocavirus; KIPyV, KI polyomavirus; WUPyV, WU polyomavirus.

Discussion

In the present study, the prevalences of hBoV, KIPyV, and WUPyV in PICU patients with LRTI (n = 78) or without LRTI (n = 83) were similar (5.1%, 0%, 2.6%; and 4.8%, 4.8%, 2.4%, respectively). Most hBoV- and KIPyW-positive LRTI patients were co-infected with other viruses. One LRTI patient with a hBoV single infection was identified. In this patient, hBoV was present in a high quantity.

Two limitations of our study deserve further discussion. First, LRTI patients were younger than controls (LRTI, 100% <5 years; controls, 40% >5 years). However, the positivity rates for hBoV, KIPyV, and WUPyV in control children <5 years were similar to rates of control children >5 years of age (6/49 vs. 3/34, respectively). Hence, the influence of this limitation is likely minor. Studies have also shown that the highest incidence of KIPyV/WUPyV infection occurs in children ≈ 1 year of age, slightly older than the children in our LRTI group (13-15).
The young age of the LRTI group may have resulted in a lower than expected positivity rate

for this group. Second, all LRTI patients had nasopharyngeal aspirates taken; however, 68.7% of controls had provided nasopharyngeal aspirates. HBoV and KIPyV/WUPyV infections were more common in controls who had nasopharyngeal aspirate samples taken than in those who had throat swab samples taken (8/57 vs. 1/26). This difference in positivity for nasopharyngeal aspirates strengthens our conclusion that these viruses are not found more frequently in PICU children with LRTI.

Sampling errors make precise quantification of viral loads difficult. Nevertheless, in the LRTI group, low Ct values, which indicate high viral loads, were found in nasopharyngeal samples taken from 3 patients infected with hBoV (Ct ≈ 15) and from 1 patient infected with WUPyV (Ct = 19). Ct values found in nasopharyngeal samples from patients in the control group were much higher, 32–39. A possible explanation for this difference is that high viral loads in the young LRTI population represent symptomatic primary infection, whereas the low viral load in the older controls might represent asymptomatic long-term shedding. Further studies are needed to show the clinical implications of infections with these viruses.

Prevalences of hBoV, KIPyV, and WUPyV infections in children in the PICU is low (≈<5% for LRTI patients and controls), and these agents are unlikely to be a major cause of LRTI at the PICU. However, hBoV might be pathogenic in some PICU patients because 1 person with a hBoV single infection in a high quantity was identified. Further studies using quantitative viral detection are needed to investigate the probability that hBoV, KIPyV, and WUPyV represent etiologic agents of LRTI.

Acknowledgements

We thank Marco Viveen, Ramona Zenhorst, and Ilse Overdevest for technical assistance; Niels Lastdrager for reviewing electronic charts; and Idse Visser for providing PaO2/FiO2 ratios. This work was supported by an “Alexandre Suerman program” MD/PhD grant from the University Medical Center Utrecht, Utrecht, the Netherlands.

Reference List

(1) van de Pol AC, Wolfs TF, Jansen NJ, van Loon AM, Rossen JW. Diagnostic value of real-time polymerase chain reaction to detect viruses in young children admitted to the paediatric intensive care unit with lower respiratory tract infection. Crit Care 2006;10(2):R61.

(2) Allander T, Tammi MT, Eriksson M, Bjerkner A, Tiveljung-Lindell A, Andersson B. Cloning of a human parvovirus by molecular screening of respiratory tract samples. Proc Natl Acad Sci U S A 2005 September 6;102(36):12891-6.

(3) Allander T, Andreasson K, Gupta S, Bjerkner A, Bogdanovic G, Persson MA, Dalianis T, Ramqvist T, Andersson B. Identification of a third human polyomavirus. J Virol 2007 April;81(8):4130-6.

(4) Gaynor AM, Nissen MD, Whiley DM, Mackay IM, Lambert SB, Wu G, Brennan DC, Storch GA, Sloots TP, Wang D. Identification of a novel polyomavirus from patients with acute respiratory tract infections. PLoS Pathog 2007 May 4;3(5):e64.

(5) McIntosh K. Human bocavirus: developing evidence for pathogenicity. J Infect Dis 2006 November 1;194(9):1197-9.

(6) Sloots TP, Whiley DM, Lambert SB, Nissen MD. Emerging respiratory agents: new viruses for old diseases? J Clin Virol 2008 July;42(3):233-43.

(7) van der Zalm MM, Rossen JW, van Ewijk BE, Wilbrink B, van Esch PC, Wolfs TF, van der Ent CK. Prevalence and pathogenicity of WU and KI polyomaviruses in children, the Netherlands. Emerg Infect Dis 2008 November;14(11):1787-9.

(8) Arnold JC, Singh KK, Spector SA, Sawyer MH. Human bocavirus: prevalence and clinical spectrum at a children's hospital. Clin Infect Dis 2006 August 1;43(3):283-8.

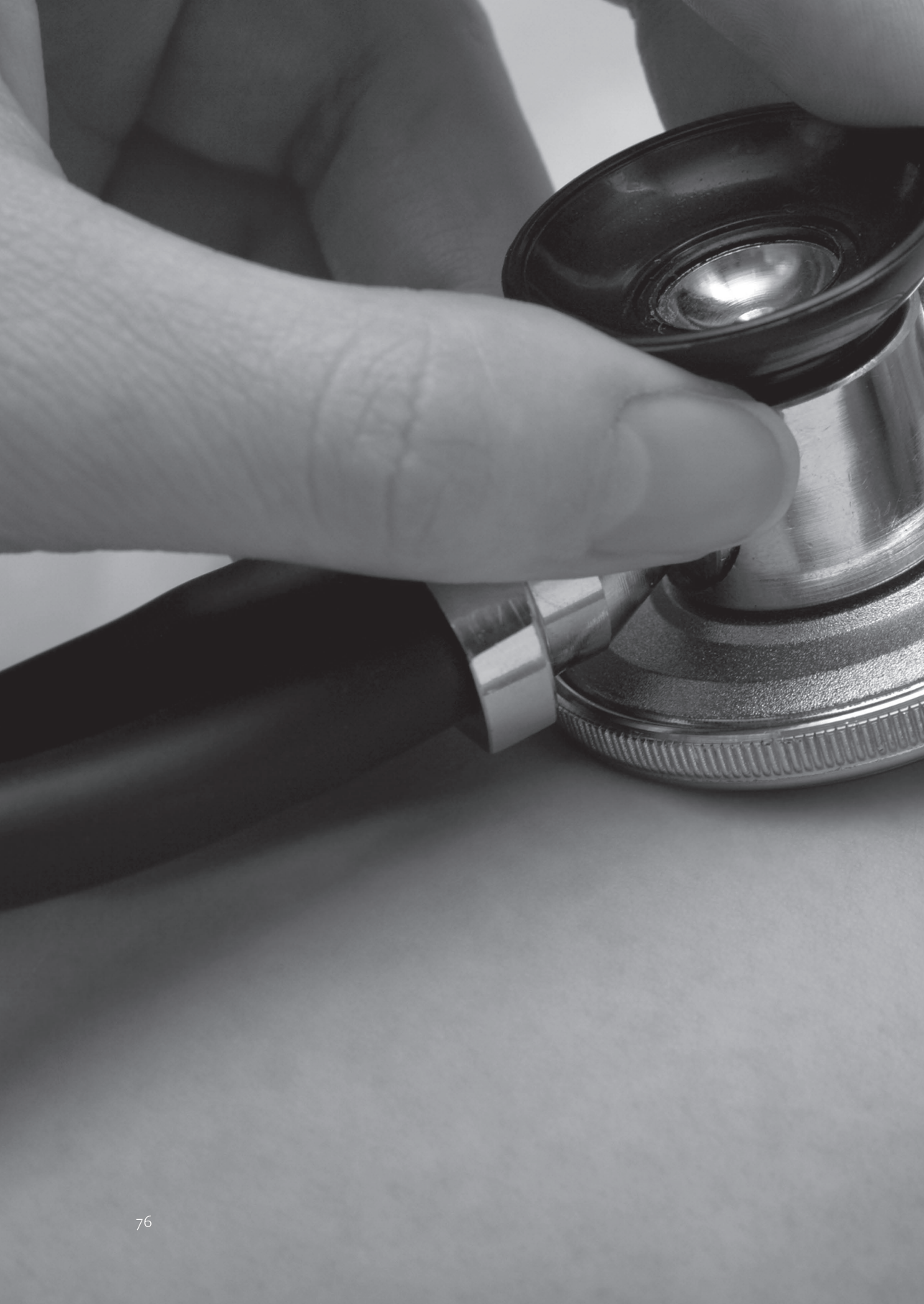
(9) van de Pol AC, van Loon AM, Wolfs TF, Jansen NJ, Nijhuis M, Breteler EK, Schuurman R, Rossen JW. Increased detection of respiratory syncytial virus, influenza viruses, parainfluenza viruses, and adenoviruses with real-time PCR in samples from patients with respiratory symptoms. J Clin Microbiol 2007 July;45(7):2260-2.

(10) van Elden LJ, van Loon AM, van der BA, Hendriksen KA, Hoepelman AI, van Kraaij MG, Schipper P, Nijhuis M. Applicability of a real-time quantitative PCR assay for diagnosis of respiratory syncytial virus infection in immunocompromised adults. J Clin Microbiol 2003 September;41(9):4378-81.

(11) Allander T, Jartti T, Gupta S, Niesters HG, Lehtinen P, Osterback R, Vuorinen T, Waris M, Bjerkner A, Tiveljung-Lindell A, van den Hoogen BG, Hyypia T, Ruuskanen O. Human bocavirus and acute wheezing in children. Clin Infect Dis 2007 April 1;44(7):904-10.

(12) Bialasiewicz S, Whiley DM, Lambert SB, Gould A, Nissen MD, Sloots TP. Development and evaluation of real-time PCR assays for the detection of the newly identified KI and WU polyomaviruses. J Clin Virol 2007 September;40(1):9-14.

(13) Abed Y, Wang D, Boivin G. WU polyomavirus in children, Canada. Emerg Infect Dis 2007 December;13(12):1939-41.
(14) Han TH, Chung JY, Koo JW, Kim SW, Hwang ES. WU polyomavirus in children with acute lower respiratory tract infections, South Korea. Emerg Infect Dis 2007 November;13(11):1766-8.
(15) Norja P, Ubillos I, Templeton K, Simmonds P. No evidence for an association between infections with WU and KI polyomaviruses and respiratory disease. J Clin Virol 2007 December;40(4):307-11.



Impact on patient management

6

7

CHAPTER 6

Does real-time PCR for respiratory viruses change antibiotic use in children admitted at the pediatric intensive care unit with lower respiratory tract infection?

Alma C. van de Pol
Tom F.W. Wolfs
Carline E.A. Tacke
Cuno S.P. Uiterwaal
Johannes Forster
Anton M. van Loon
Jan L.L. Kimpen
John W.A. Rossen
Nicolaas J.G. Jansen

Submitted

Abstract

Objectives

To determine whether respiratory virus real-time PCR, in addition to direct immunofluorescence (DIF), changes antibiotic use in ventilated children with lower respiratory tract infection (LRTI).

Methods

We first performed a multicenter paper case study, followed by a prospective study, to evaluate the impact of PCR in addition to DIF in children ventilated for LRTI. In the prospective study children (≤5 years) were tested by DIF and PCR. Positive DIF results were reported at the end of the first working-day. Physicians reported antibiotic treatment on the second working-day. After informing them of the PCR result antibiotic treatment was reevaluated.

Results

The multicenter paper case study (94 respondents) showed that PCR decreased antibiotic use ($p<0.001$). In the prospective real-life study 38 children were included, of which 19 (50%) were positive by DIF. Twelve (63%) of the 19 DIF-negative patients were treated with antibiotics before revealing the PCR result; the PCR test was positive in 9 out of 12. Revealing PCR results did not alter antibiotic treatment. In the 7 DIF-negative patients not given antibiotics, the PCR test was positive.

Conclusions

In contrast to their responses to our multicenter paper case study, in real-life PICU physicians did not let their antibiotic prescription be influenced by real-time PCR for respiratory viruses in children ventilated for LRTI.

Introduction

Lower respiratory tract infection (LRTI) in young children is a common reason for admission to the pediatric intensive care unit (PICU). LRTI is often of viral origin (1-3), in which case antibiotic treatment would be unnecessary. However, the differentiation between viral, bacterial or mixed infections is clinically impossible and most children with LRTI are therefore treated empirically with antibiotics (4;5). The usefulness of rapid respiratory viral testing in the work-up of LRTI is unclear (6). The limited number of studies evaluating the effect of rapid testing on antibiotics use yielded contradictory results (7-12). The impact of viral testing in the PICU setting has not been investigated previously.

Rapid viral testing in the hospital setting has traditionally been performed with direct immunofluorescence tests (DIF). Currently, molecular amplification tests such as real-time polymerase chain reaction (PCR) have become the new golden standard for the detection of respiratory viruses because of their increased sensitivity (13;14). Real-time PCR assays have been shown to be quick and reliable in the PICU, leading to a large increase in diagnostic yield of respiratory viruses (15;16). At present, the high costs of PCR assays are considered a disadvantage.

The aim of our study was to evaluate whether a viral diagnosis obtained by the more sensitive real-time PCR as compared to DIF, would impact antibiotic prescription in children with LRTI at the PICU. To answer this question we first performed a multicenter paper case study among PICU physicians. Subsequently, we conducted a 2-year prospective real-life study at our PICU.

Methods

Multicenter paper case study

Paper cases

Questionnaires consisted of five case descriptions, constructed from clinical data from five infants with typical signs of LRTI ventilated at our PICU in 2004. The infants were selected because they had a negative or indeterminate DIF test and a positive real-time PCR result. After each case description the following question was asked: "Would you start/continue antibiotic therapy for this child?". Questions were first asked with the real-time PCR result concealed, and

the same questions were repeated after disclosing the real-time PCR result. Anonymity of the respondents was assured. The questionnaire was pilot-tested to assure clarity and coherence, and it was subsequently modified in response to the pilot test results. The order of cases was random in the different questionnaires. Due to the low burden of the survey, ethical approval/ informed consent was not needed according to the institutional review board of our hospital.

Respondents

A total of six PICU's from three large Dutch medical centers (University Medical Center Utrecht, Amsterdam Medical Center, Erasmus Medical Center Rotterdam) and three large German medical centers (University Medical Center Freiburg, Universitätsklinikum Mainz, Charité Berlin) participated in this multicenter study. A senior pediatric intensivist of each PICU listed the PICU physicians involved in the care of infants with LRTI at their PICU. Physicians were individually approached by the investigator or the pediatric intensivist for a response.

Prospective real-life study

Patients

Ventilated patients under 5 years old admitted with LRTI to the PICU of the Wilhelmina Children's Hospital, who had a DIF test performed as part of routine investigation, were enrolled from October 2006 through October 2008. The Wilhelmina Children's Hospital is a tertiary care medical center with a 16 bed PICU facility. It serves as a referral center for the central part of the Netherlands. Exclusion criteria were asthma exacerbation, immune compromised state, indication for antibiotics other than LRTI, and repeated PICU admission for LRTI during the study period. Baseline clinical data were obtained from medical charts using standardized forms (gender, age, symptom onset, disease severity, antibiotics before admission, preterm birth, underlying illness, duration of ventilation, length of PICU stay, white blood count and C-reactive protein). Underlying illness was defined as chronic pulmonary disease, congenital heart disease, neurological disease or gastrointestinal disease (17). For the assessment of illness severity the oxygenation index (OI) was calculated 12 hours after admission (OI = fraction of inspired oxygen x mean airway pressure x 100 / partial oxygen pressure).

Viral testing

Nasopharyngeal aspirates were taken by trained nursing staff the morning of the first working-day after admission (working-days: Monday through Friday). Specimens were examined with DIF and real-time PCR as previously described (16;18-20). In short, part of the sample was subjected to DIF assays to detect respiratory syncytial virus (RSV), influenza viruses, parainfluenza

viruses 1–3 and adenoviruses using Imagen kits (DaKo, Glostrup, Denmark), in accordance with the manufacturer's recommended protocol. Indeterminate DIF results were considered negative for the analyses. The remaining material was used for real-time PCR testing for RSV, influenza viruses, parainfluenza viruses, adenoviruses, human rhinoviruses, coronaviruses, human metapneumovirus and Mycoplasma pneumoniae. During the second year the newly detected human bocavirus was added to the panel (21;22). Briefly, after nucleic acid extraction using the MagNA pure LC nucleic acid isolation system (Roche Diagnostics, Basel, Switzerland) amplification was carried out in a 25-µl reaction mixture on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The positive control for human bocavirus was provided by T. Allander, Karolinska Institute, Stockholm, Sweden. Internal control viruses were used to monitor for efficient extraction and amplification.

Patient management survey

Patient care of enrolled patients was left at the discretion of the attending PICU physician. Results of the DIF assays had a turn-around time of approximately five hours, and positive results were reported at the end of the first working-day to the attending clinician. Results of the real-time PCR assays had a turn-around time between 16 and 24 hours and the results were reported at the beginning of the second working-day to the investigator only. The investigator approached the attending physician before revealing the PCR result, using a questionnaire with the following questions: (1) "What is the patients' suspected clinical diagnosis?" (To be answered with checkboxes for viral bronchiolitis, viral pneumonia, and bacterial pneumonia), and (2) "Is the patient treated with antibiotics?". Subsequently, the physician was provided with the PCR result, and one hour later the questions were repeated. Finally, PICU physicians were asked what the reason was for (dis)continuing antibiotics. Since the survey did not involve a change in patient management, ethical approval/informed consent was not needed according to the institutional review board of our hospital.

Results

Multicenter paper case study

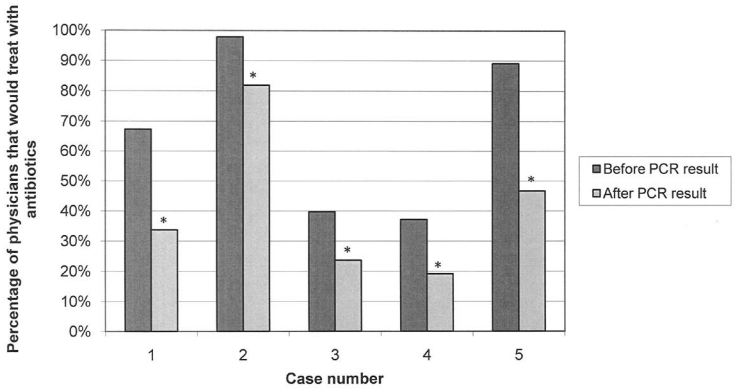
Respondents

Ninety-four PICU physicians (20 from Utrecht, 12 from Amsterdam, 16 from Rotterdam, 17 from Freiburg, 12 from Mainz, and 17 from Berlin) completed the questionnaire.

Antibiotic use

Before disclosure of the real-time PCR result, the percentage of PICU physicians that would treat the five infants with antibiotics was 37%-98% (range for the five cases; Figure 1). After disclosure of the real-time PCR result the percentage of physicians that would treat the infants with antibiotics decreased to 19%-82% (McNemar test: p<0.001 for all cases). The absolute reduction was largest for case 1 and 5 (37% and 42%). Although the percentage of antibiotic use pre-PCR test differed between centers, the reduction of antibiotic use following PCR was similar in the different centers.

Figure 1 Antibiotic use before and after disclosure of the real-time PCR results in the paper case study



** Difference McNemar test p<0.001*

Prospective real-life study

Patients

Thirty-eight children were enrolled in this study. Twenty (53%) children were clinically suspected of viral bronchiolitis, 9 (24%) of viral pneumonia and 9 (24%) of bacterial pneumonia. Demographics and clinical characteristics of the enrolled patients are provided in Table 1.

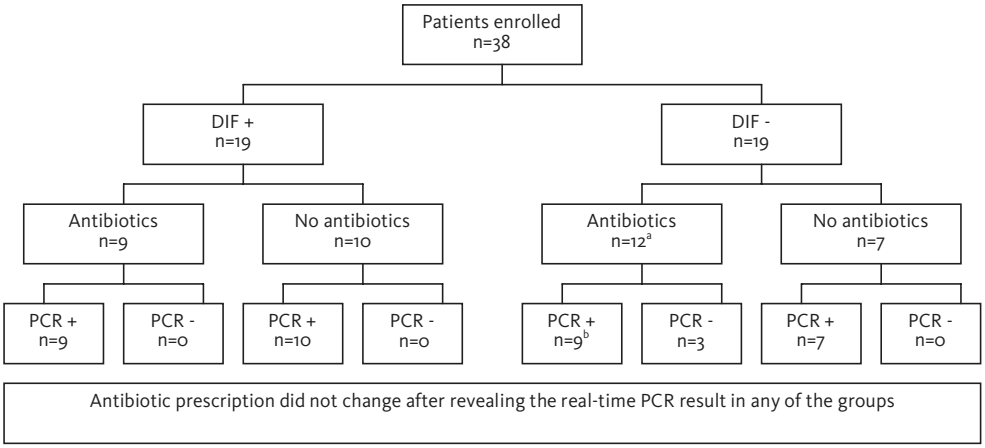
Viral test results

Nineteen patients were positive with DIF (50%), all for RSV (Figure 2). Real-time PCR confirmed all 19 positive DIF results and detected 7 additional viruses in 6 of them (Table 2). The remaining 19 patients were DIF negative. In 16 of the 19 DIF negative patients real-time PCR detected a total of 20 viruses. In 3 (8%) patients with LRTI no virus could be identified either by DIF or by PCR. Therefore, an infection with one or more viruses was detected in 35 of 38 patients (92%).

Antibiotic prescription before and after the PCR result

In the group of 19 DIF positive patients, 9 (47%) were treated with antibiotics before the real-time PCR result was made available. Antibiotic prescription did not change after revealing the concordant positive real-time PCR results (Figure 2).

Figure 2 Flow-chart of viral test results and antibiotic prescription of patients enrolled in the Prospective real-life study



[a] *Twelve patients that were negative by direct immunofluorescence were treated with antibiotics.*

[b] *Nine out of twelve patients that were negative by direct immunofluorescence and treated with antibiotics were subsequently positive by real-time PCR. These are the patients in which antibiotic prescription is most likely to be changed.*

In the group of 19 DIF negative patients, 12 (63%) patients were treated with antibiotics before the PCR result was made available, and real-time PCR was positive in 9 of these 12 patients. In 1 of these 9 patients the suspected clinical diagnosis was changed from bacterial to viral pneumonia. In none of the 9 patients the positive real-time PCR result prompted the PICU physicians to discontinue antibiotics. When asked for the reason to continue antibiotics, physicians listed (a) clinical suspicion of bacterial superinfection (ill-appearance, high ventilator settings, high C-reactive protein levels; n=7) and (b) waiting for the final result of the bacterial cultures (n=2). Finally, in the 7 DIF negative patients that were not treated with antibiotics, a negative real-time PCR result could have prompted physicians to start antibiotics. However, all 7 patients were found to be positive by real-time PCR for respiratory viruses; i.e. real time PCR confirmed the clinical suspicion of a viral infection and no antibiotics were started.

Discussion

The present study investigated the impact of respiratory virus real-time PCR, in addition to DIF, on antibiotic prescription in ventilated PICU children with LRTI, first in a paper case study and then in a prospective real-life study. Although physicians indicated that real-time PCR would have an effect on antibiotic use in the paper case study, this effect was not found in practice in the prospective study.

A variety of viral tests are currently used in the diagnostic work-up of children with LRTI at the PICU. The clinical utility of none of these tests has clearly been demonstrated (6). In recent years, conventional rapid viral tests such as antigen detection by DIF are being replaced by more sensitive, molecular assays such as real-time PCR. This has led to an increase in the detection of viruses (16;23-25), with a substantial increase in costs (18). To determine if real time PCR theoretically could influence daily practice at the PICU first a multicenter paper case study was performed. Since the paper case study indicated that the availability of PCR result would reduce antibiotic usage, a 2-years prospective study was undertaken at a single center to test the impact of real-time PCR in real-life.

Two limitations of our study deserve further discussion. First, the number of evaluated PICU children in the prospective real-life study was relatively small. However, real-time PCR did not influence antibiotic prescription in one single patient. Therefore, we believe that the inclusion of additional patients is unlikely to influence our observation that the impact of real-time PCR is minor. Second, the turn around time of real-time PCR in our study was between 16 and 24 hours and PCR was performed on work-days, so the results were available only at the second working-day. Possibly, if results had been available earlier this could have had more effect on antibiotic prescription practices (physicians will probably be more reluctant to stop antibiotic treatment than not to start it). However, we aimed to evaluate the impact of real-time PCR in daily practice, and in most hospitals both DIF and real-time PCR are only performed on working-days (i.e. Monday-Friday) (26).

In agreement with previous studies we showed that real-time PCR increased the diagnostic yield considerably when compared to DIF. The proportion of virus-infected children increased from 50% (DIF) to 92% (real-time PCR), and the number of viruses from a total of 19 (all RSV) to 46 (8 different types). This increased diagnostic yield was not, however, accompanied by a decrease in antibiotic prescription. This finding is in contrast with our paper case study in which real-time PCR decreased the percentage of antibiotic description by 16-42% (absolute reduction; $p<0.001$ for all cases). Apparently, the treatment of paper patients is not the same as treating patients in daily PICU practice, in which physicians seem to prefer to stay on the safe

side. In daily practice, physicians seem to make clinical decisions on other grounds than results from diagnostic investigations. Reasons for not stopping antibiotic treatment in PCR positive patients included clinical suspicion of a bacterial superinfection, and uncertainty about the final results of bacterial cultures.

Previous studies on the impact of rapid viral testing on antibiotic use in children have been published. Our results are in agreement with Doan et al and Poehling et al, who did not find a reduction in antibiotic prescription in children randomized to have rapid testing performed at the emergency department (8;10). Our results are in contrast with studies from Bonner et al, Noyola et al, Sharma et al, and Woo at al, who found that physicians prescribed less antibiotics in patient groups tested with a rapid viral test (7;9;11;12) . These studies were all performed at the emergency department or at pediatric wards, and not at the PICU, and assessed different age groups. It is likely that physicians are more reluctant to withhold antibiotic treatment in the severely ill population of LRTI patients at the PICU. This is reflected in our population by the finding that LTRI in 29 out of 38 patients (76%) were suspected to be of viral origin of disease, yet 12 of these 29 patients received antibiotics (41%).

In addition, virtually all LRTI patients at our PICU (35 out of 38; 92%) were found to be positive by real-time PCR for one or more viruses. This means that in our PICU population virus-positivity does not help clinicians anymore to differentiate between those patients who qualify for antibiotics from those who could safely be treated without antibiotics. Therefore, we propose that future real-time PCR studies should be directed to settings were nót all patients are found to harbour viruses, such as the population of children with less severe respiratory tract infection in the community (8).

Limiting antibiotic prescriptions in patients with viral LRTI is not the only objective of performing respiratory real-time PCR assays. Real-time PCR is valuable as a very sensitive tool to further explore the clinical presentation and impact of respiratory viruses as well as their epidemiology and it will become an even more important diagnostic tool when antiviral treatments become available. However, when real-time PCR in LRTI patients at the PICU does not affect daily practice, this test could be left out of the diagnostic work-up and costs could be saved.

In conclusion, real time PCR, in addition to DIF testing, has no impact on the prescription of antibiotics in ventilated LRTI patients at the PICU in daily practice. This is in contrast to the response of physicians on paper case patients. This demonstrates that physicians seem reluctant to stop antibiotic prescriptions in the severely ill population of LRTI patients at the PICU.

Acknowledgements

We thank Dr. M. Krüger (University Medical Center Freiburg, Germany), Dr. J.-H. Höpner (Universitätsklinikum Mainz, Germany), Dr. S. Henning (Charité Berlin, Germany), Dr. L.J.A. Corel (Erasmus Medical Center Rotterdam, The Netherlands), and Dr. J.B.M. van Woensel (Amsterdam Medical Center, The Netherlands) for their help with the distribution of the questionnaires. We thank Dr. I.C. Albers, Dr. A.C. Blank, and Dr. M.W. Freund (University Medical Center Utrecht) for the translation of the German questionnaires. The study was supported by an "Alexandre Suerman program" MD/PhD grant from the University Medical Center Utrecht, The Netherlands.

Reference List

(1) Shay DK, Holman RC, Newman RD, Liu LL, Stout JW, Anderson LJ. Bronchiolitis-associated hospitalizations among US children, 1980-1996. JAMA 1999;282:1440-6.

(2) Randolph AG, Meert KL, O'Neil ME, Hanson JH, Luckett PM, Arnold JH, et al. The feasibility of conducting clinical trials in infants and children with acute respiratory failure. Am J Respir Crit Care Med 2003;167:1334-40.

(3) Mansbach JM, Clark S, Christopher NC, LoVecchio F, Kunz S, Acholonu U, et al. Prospective multicenter study of bronchiolitis: predicting safe discharges from the emergency department. Pediatrics 2008;121:680-8.

(4) Kneyber MC, van Woensel JB, Uijtendaal E, Uiterwaal CS, Kimpen JL. Azithromycin does not improve disease course in hospitalized infants with respiratory syncytial virus (RSV) lower respiratory tract disease: a randomized equivalence trial. Pediatr Pulmonol 2008;43:142-9.

(5) Purcell K, Fergie J. Driscoll Children's Hospital respiratory syncytial virus database: risk factors, treatment and hospital course in 3308 infants and young children, 1991 to 2002. Pediatr Infect Dis J 2004;23:418-23.

(6) Bordley WC, Viswanathan M, King VJ, Sutton SF, Jackman AM, Sterling L, et al. Diagnosis and testing in bronchiolitis: a systematic review. Arch Pediatr Adolesc Med 2004;158:119-26.

(7) Bonner AB, Monroe KW, Talley LI, Klasner AE, Kimberlin DW. Impact of the rapid diagnosis of influenza on physician decision-making and patient management in the pediatric emergency department: results of a randomized, prospective, controlled trial. Pediatrics 2003;112:363-7.

(8) Doan QH, Kissoon N, Dobson S, Whitehouse S, Cochrane D, Schmidt B, et al. A randomized, controlled trial of the impact of early and rapid diagnosis of viral infections in children brought to an emergency department with febrile respiratory tract illnesses. J Pediatr 2009;154:91-5.
(9) Noyola DE, Demmler GJ. Effect of rapid diagnosis on management of influenza A infections. Pediatr Infect Dis J 2000;19:303-7.
(10) Poehling KA, Zhu Y, Tang YW, Edwards K. Accuracy and impact of a point-of-care rapid influenza test in young children with respiratory illnesses. Arch Pediatr Adolesc Med 2006;160:713-8.
(11) Sharma V, Dowd MD, Slaughter AJ, Simon SD. Effect of rapid diagnosis of influenza virus type a on the emergency department management of febrile infants and toddlers. Arch Pediatr Adolesc Med 2002;156:41-3.
(12) Woo PC, Chiu SS, Seto WH, Peiris M. Cost-effectiveness of rapid diagnosis of viral respiratory tract infections in pediatric patients. J Clin Microbiol 1997;35:1579-81.
(13) Henrickson KJ. Advances in the laboratory diagnosis of viral respiratory disease. Pediatr Infect Dis J 2004;23:S6-10.
(14) Iwane MK, Edwards KM, Szilagyi PG, Walker FJ, Griffin MR, Weinberg GA, et al. Population-based surveillance for hospitalizations associated with respiratory syncytial virus, influenza virus, and parainfluenza viruses among young children. Pediatrics 2004;113:1758-64.
(15) Louie JK, Roy-Burman A, Guardia-Labar L, Boston EJ, Kiang D, Padilla T, et al. Rhinovirus Associated With Severe Lower Respiratory Tract Infections in Children. Pediatr Infect Dis J 2009;28:337-9.
(16) van de Pol AC, Wolfs TF, Jansen NJ, van Loon AM, Rossen JW. Diagnostic value of real-time polymerase chain reaction to detect viruses in young children admitted to the paediatric intensive care unit with lower respiratory tract infection. Crit Care 2006;10:R61.
(17) Arnold JC, Singh KK, Spector SA, Sawyer MH. Human bocavirus: prevalence and clinical spectrum at a children's hospital. Clin Infect Dis 2006;43:283-8.
(18) Oosterheert JJ, van Loon AM, Schuurman R, Hoepelman AI, Hak E, Thijsen S, et al. Impact of rapid detection of viral and atypical bacterial pathogens by real-time polymerase chain reaction for patients with lower respiratory tract infection. Clin Infect Dis 2005;41:1438-44.
(19) van de Pol AC, van Loon AM, Wolfs TF, Jansen NJ, Nijhuis M, Breteler EK, et al. Increased detection of respiratory syncytial virus, influenza viruses, parainfluenza viruses, and adenoviruses with real-time PCR in samples from patients with respiratory symptoms. J Clin Microbiol 2007;45:2260-2.
(20) van Elden LJ, van Loon AM, van der BA, Hendriksen KA, Hoepelman AI, van Kraaij MG, et al. Applicability of a real-time quantitative PCR assay for diagnosis of respiratory syncytial virus infection in immunocompromised adults. J Clin Microbiol 2003;41:4378-81.

(21) Allander T, Tammi MT, Eriksson M, Bjerkner A, Tiveljung-Lindell A, Andersson B. Cloning of a human parvovirus by molecular screening of respiratory tract samples. Proc Natl Acad Sci U S A 2005;102:12891-6.
(22) van de Pol AC, Wolfs TF, Jansen NJ, Kimpen JL, van Loon AM, Rossen JW. Human bocavirus and KI/WU polyomaviruses in pediatric intensive care patients. Emerg Infect Dis 2009;15:454-7.
(23) Arden KE, McErlean P, Nissen MD, Sloots TP, Mackay IM. Frequent detection of human rhinoviruses, paramyxoviruses, human coronaviruses, and bocavirus during acute respiratory tract infections. J Med Virol 2006;78:1232-40.
(24) Arnold JC, Singh KK, Spector SA, Sawyer MH. Undiagnosed respiratory viruses in children. Pediatrics 2008;121:e631-e637.
(25) Jennings LC, Anderson TP, Werno AM, Beynon KA, Murdoch DR. Viral etiology of acute respiratory tract infections in children presenting to hospital: role of polymerase chain reaction and demonstration of multiple infections. Pediatr Infect Dis J 2004;23:1003-7.
(26) Landry ML, Cohen S, Ferguson D. Real-time PCR compared to Binax NOW and cytospin-immunofluorescence for detection of influenza in hospitalized patients. J Clin Virol 2008;43:148-51.

CHAPTER 7

Transmission of RSV at the PICU: a prospective study using real-time PCR

Alma C. van de Pol
John W.A. Rossen
Tom F.W. Wolfs
Els K. Breteler
Jan L.L. Kimpen
Anton M. van Loon
Nicolaas J.G. Jansen

Clin Microbiol Infect 2009, in press

Abstract

Transmission of respiratory syncytial virus (RSV) from children with lower respiratory tract infection (LRTI) at a paediatric intensive-care unit (PICU) was examined using a highly sensitive real-time PCR. Twenty-four children with RSV LRTI were admitted during the study period (total days of potential transmission: 239). Forty-eight RSV-negative patients were followed up for RSV acquisition every 5 days (total days of exposure: 683). No single RSV transmission was documented with this highly sensitive diagnostic method. Therefore, routine infection control measures of LRTI patients seem to be adequate to prevent RSV transmission at the PICU.

Introduction

Community-acquired respiratory syncytial virus (RSV) lower respiratory tract infection (LRTI) is a common reason for admission to a paediatric intensive-care unit (PICU) (1-3). RSV has been shown to be transmitted to other patients, causing nosocomial infections with significant morbidity (4). Research on the frequency of transmission of RSV at the PICU has shown contradictory results (5-8). RSV detection has traditionally been performed with direct immunofluorescence (DIF) and viral culture. Real-time PCR is a more sensitive detection method than DIF and viral culture (9), but is also more expensive. This is the first study using real-time PCR to prospectively reveal RSV transmission from children with RSV LRTI to other children admitted to a PICU. To confirm that RSV acquisition was nosocomial, patients were assessed for asymptomatic carriage of RSV upon admission to the PICU, and subsequently every 5 days.

Methods

During the RSV season of 2005–2006, 24 patients with RSV LRTI were admitted to the PICU of Wilhelmina Children's Hospital. Every 5 days, it was determined whether patients were still shedding virus. If a patient with RSV LRTI was admitted to the unit, other patients without LRTI who were present in the unit or admitted to the unit were tested for RSV. This surveillance was continued during all periods in which patients were shedding RSV ('RSV-positive periods'). Exclusion criteria were as follows: (i) post-head trauma status; (ii) discomfort of sampling was deemed to be unethical; and (iii) PICU stay <12 h/weekend only. As RSV surveillance of non-LRTI

patients was part of routine infection control measures; according to the Medical Ethical Research Council of the institution, there was no need for patient consent/ethical approval. Every child without LRTI who stayed at the PICU for >3 days was eligible for follow-up. For logistic reasons, all children at the unit were sampled on the same day ('sample day'), every 5 days.

RSV detection was performed by examining nasopharyngeal aspirates in intubated children and throat swabs in extubated children. Specimens were examined with DIF, viral culture and real-time PCR as previously described (9-11). In short, part of the sample was inoculated onto HEp cells for viral culture, and examined twice weekly for cytopathogenic effect. A second aliquot was used for DIF detection (Imagen; DaKo, Glostrup, Denmark). DIF was not performed on throat swabs. The last part of the sample was used for real-time PCR (9-11). Briefly, after nucleic acid extraction (MagNA pure LC; Roche Diagnostics, Basel, Switzerland), cDNA was synthesized using MultiScribe reverse transcriptase and random hexamers (both from Applied Biosystems, Foster City, CA, USA). Finally, amplification was carried out in duplicate in a 25-µL reaction mixture. Murine encephalomyocarditis virus was used as an internal control virus to monitor for efficient extraction, cDNA synthesis, and amplification (12). As transmission of RSV occurs mainly through contact with objects rather than through aerosols, routine infection control measures for LRTI patients included rigorous hand hygiene, together with gloving and gowning.

Results

During the study period, 24 RSV LRTI patients were admitted, staying for a mean of 10 days (standard deviation: 4.4 days). Follow-up samples remained positive for a mean of 7 days (standard deviation: 4.8 days). In total, RSV LRTI patients were shedding virus for 239 days. During the RSV-positive periods, 87 children were tested for RSV upon admission (Figure 1; 58 nasopharyngeal aspirates and 29 throat swabs).

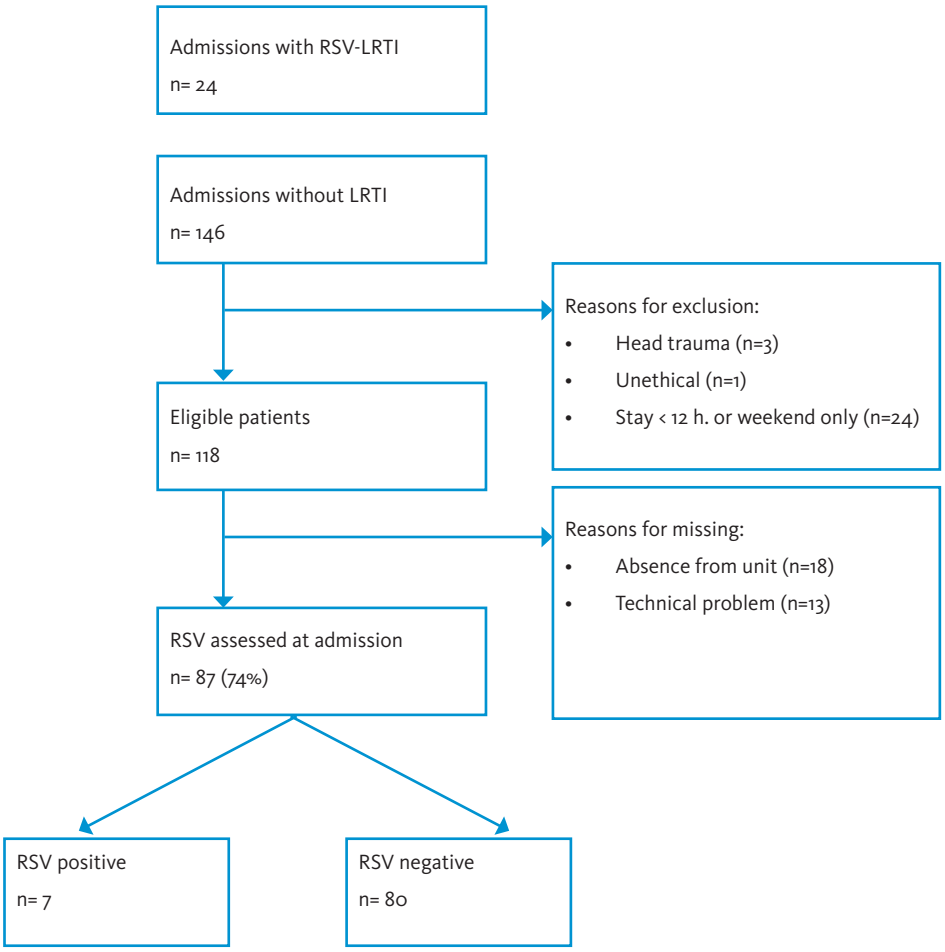
Seven of the 87 examined patients were found to be asymptomatic carriers of RSV (four identified by PCR and DIF, and three identified by PCR only). Age and length of stay were diverse among these patients. Most children had a significant medical history, and were admitted for surgical reasons. Notably, two children had suffered from an RSV infection 1 week prior to admission to the PICU. However, none of these patients showed any sign of LRTI during their entire stay at the PICU.

Fifty-eight patients stayed at the PICU for >3 days. Forty-eight patients (83%) stayed long enough to be present during at least one 'sample day', and follow-up results were therefore available.

In total, non-LRTI patients were exposed for 683 days to RSV-shedding LRTI patients. None of the patients was found to acquire RSV during follow-up, regardless of whether real-time PCR, viral culture or DIF tests were analysed.

For the detection of RSV, real-time PCR was the viral test with the highest positivity rate. With real-time PCR, 31 RSV-positive patients were detected, whereas viral culture and DIF revealed 13 and 21 RSV-positive patients, respectively.

Figure 1 Flow of patients included in the study during the respiratory syncytial virus (RSV)-positive periods.



LRTI, lower respiratory tract infection.

Discussion

Children with RSV LRTI admitted at the PICU in the RSV season of 2005-2006 were not found to transmit RSV to patients without RSV LRTI, neither when tested by DIF, nor by viral culture or real-time PCR.

Several limitations of our study warrant further discussion. First, it was not possible to sample all eligible PICU patients to determine asymptomatic carriership, owing to early discharge, absence from the unit for additional tests or surgery, or technical problems. Second, owing to the study design, in which the entire PICU population was sampled on the same day ('sample day'), every 5 days, there was no follow-up sample for some patients who stayed for >3 days at the PICU. Third, RSV transmission was assessed only when patients with RSV LRTI were admitted to the PICU.

An unexpected finding of the study was that seven patients without LRTI were found to carry RSV upon admission. These asymptomatic RSV patients can shed RSV and cause nosocomial infections, especially because they are not recognized and no infection control measures are taken. RSV is believed to be shed for up to 2 weeks after symptoms occur; however, most of the literature on RSV shedding is based on viral culture results and not on more sensitive tests such as real-time PCR (13). Thus, the length of time for which RSV patients shed RSV is not obvious. Therefore, the impact of asymptomatic shedders at the PICU must be the focus of further study, and these studies must maintain RSV surveillance during periods in which the PICU seems to be 'RSV-free'.

In conclusion, children with RSV LRTI admitted to the PICU during the RSV season were not found to transmit RSV to patients without LRTI, even though a highly sensitive real-time PCR assay was used. Therefore, routine infection control measures for LRTI patients seem to be adequate to prevent RSV transmission at the PICU.

Acknowledgements

This work was presented in part at the Dutch Association for Paediatrics Meeting 2007 in Veldhoven, The Netherlands. N. de Jong is kindly acknowledged for his contribution to the collection of samples. The study was supported by an 'Alexandre Suerman program' MD/PhD grant from the University Medical Center Utrecht, The Netherlands. A. C. van de Pol received a research grant in 2008 from the European Society of Clinical Microbiology and Infectious Disease (ESCMID).

Reference List

(1) Bosis S, Esposito S, Niesters HG, Zuccotti GV, Marseglia G, Lanari M, Zuin G, Pelucchi C, Osterhaus AD, Principi N. Role of respiratory pathogens in infants hospitalized for a first episode of wheezing and their impact on recurrences. Clin Microbiol Infect 2008 July;14(7):677-84.

(2) Grondahl B, Puppe W, Weigl J, Schmitt HJ. Comparison of the BD Directigen Flu A+B Kit and the Abbott TestPack RSV with a multiplex RT-PCR ELISA for rapid detection of influenza viruses and respiratory syncytial virus. Clin Microbiol Infect 2005 October;11(10):848-50.

(3) Randolph AG, Meert KL, O'Neil ME, Hanson JH, Luckett PM, Arnold JH, Gedeit RG, Cox PN, Roberts JS, Venkataraman ST, Forbes PW, Cheifetz IM. The feasibility of conducting clinical trials in infants and children with acute respiratory failure. Am J Respir Crit Care Med 2003 May 15;167(10):1334-40.

(4) Langley JM, LeBlanc JC, Wang EE, Law BJ, MacDonald NE, Mitchell I, Stephens D, McDonald J, Boucher FD, Dobson S. Nosocomial respiratory syncytial virus infection in Canadian pediatric hospitals: a Pediatric Investigators Collaborative Network on Infections in Canada Study. Pediatrics 1997 December;100(6):943-6.

(5) Berner R, Schwoerer F, Schumacher RF, Meder M, Forster J. Community and nosocomially acquired respiratory syncytial virus infection in a German paediatric hospital from 1988 to 1999. Eur J Pediatr 2001 September;160(9):541-7.

(6) Gagneur A, Sizun J, Vallet S, Legr MC, Picard B, Talbot PJ. Coronavirus-related nosocomial viral respiratory infections in a neonatal and paediatric intensive care unit: a prospective study. J Hosp Infect 2002 May;51(1):59-64.

(7) Hornstrup MK, Trommer B, Siboni K, Nielsen B, Kamper J. Nosocomial respiratory syncytial virus infections in a paediatric department. J Hosp Infect 1994 March;26(3):173-9.

(8) Thorburn K, Kerr S, Taylor N, van Saene HK. RSV outbreak in a paediatric intensive care unit. J Hosp Infect 2004 July;57(3):194-201.

(9) van de Pol AC, Wolfs TF, Jansen NJ, van Loon AM, Rossen JW. Diagnostic value of real-time polymerase chain reaction to detect viruses in young children admitted to the paediatric intensive care unit with lower respiratory tract infection. Crit Care 2006;10(2):R61.

(10) van de Pol AC, van Loon AM, Wolfs TF, Jansen NJ, Nijhuis M, Breteler EK, Schuurman R, Rossen JW. Increased detection of respiratory syncytial virus, influenza viruses, parainfluenza viruses, and adenoviruses with real-time PCR in samples from patients with respiratory symptoms. J Clin Microbiol 2007 July;45(7):2260-2.

(11) van Elden LJ, van Loon AM, van der BA, Hendriksen KA, Hoepelman AI, van Kraaij MG, Schipper P, Nijhuis M. Applicability of a real-time quantitative PCR assay for diagnosis of respiratory syncytial virus infection in immunocompromised adults. J Clin Microbiol 2003 September;41(9):4378-81.

(12) van Doornum GJ, Guldemeester J, Osterhaus AD, Niesters HG. Diagnosing herpesvirus infections by real-time amplification and rapid culture. J Clin Microbiol 2003 February;41(2):576-80.
(13) Hall CB. Nosocomial respiratory syncytial virus infections: the “Cold War” has not ended. Clin Infect Dis 2000 August;31(2):590-6.

Pathophysiology of RSV

8

9

CHAPTER 8

Molecular quantification of respiratory syncytial virus in the nose: a valid tool during the initial phase of infection

Alma C. van de Pol
Tom F.W. Wolfs
Anton M. van Loon
Carline E.A. Tacke
Marco C. Viveen
Nicolaas J.G. Jansen
Jan L.L. Kimpen
John W.A. Rossen
Frank E.J. Coenjaerts

Submitted

Abstract

Quantitative real-time PCR for the detection of respiratory syncytial virus (RSV) RNA is used increasingly to study the causal role of RSV in lower airway disease. The objective of our study was to validate different steps in the RNA quantification process: [1] the variation in RSV RNA load within one sample; [2] the variation in samples from patients that were sampled twice on the same day; and [3] the variation between simultaneously taken nasopharyngeal aspirates (NPAs) and tracheal aspirates (TAs). Thirty-two infants with RSV at the pediatric intensive care unit (PICU) were included. Undiluted NPAs and TAs were taken three times a week during ventilation. Intra-sample variation [1] was shown to be minimal (<2 Cycle threshold [Ct]). Intra-day variation [2] was lowest in samples with high viral loads (95% limits of agreement -3.6 to 4.8 Ct), whereas it increased in samples with loads at the lower detection limit (Ct >35) (n=138 sample-pairs from 20 patients). RSV loads in NPAs and TAs [3] were found to be most comparable during the early phase of infection (95% limits of agreement -5.2 to +5.8 Ct). Variation increased during the late phase of infection (i.e. in follow-up samples), with NPA loads remaining significantly higher than TA loads (n=138 sample-pairs from 31 patients). In conclusion, quantitative detection of RSV RNA in undiluted mucus is a reliable method to quantify viral loads. Nasal samples collected in the initial phase of infection can be used to predict RSV RNA loads in the lower airways.

Introduction

Respiratory syncytial virus (RSV) lower respiratory tract infections (LRTIs) cause significant hospitalization among young children. It is currently unclear why most children experience relatively mild symptoms, whereas a small subgroup of children develop respiratory failure, necessitating mechanical ventilation at the pediatric intensive care unit (PICU) (1;2). Quantitative RSV detection is paramount to study the relation between RSV infection and LRTI disease severity. Traditionally, quantitative detection of infectious virus has been performed using viral culture, which is labor intensive and has a low sensitivity (3;4). Currently, real-time PCR assays have become available to measure RSV RNA load, greatly expanding opportunities for respiratory viral load research (5-7).

Quantitative RSV detection is complicated by several issues. First, mucus secretions from the airways are non-homogeneous, and intra-sample variability may pose a problem. Second, the amount and composition of airway secretions over time is variable, which may lead to

intra-day variability in RSV concentration. Third, the lower respiratory tract is not easily available for sampling. Therefore, RSV load is usually detected in nasopharyngeal aspirates (NPAs), as a surrogate measure for RSV load in the lower airways. However, it is unclear whether RSV load in the upper airways reflects RSV load in the lower respiratory tract (8). The aim of our study was to validate the different steps of quantitative real-time PCR detection of RSV RNA in NPAs by evaluating [1] variation in load within respiratory samples (intra-sample variation), [2] variation in samples from patients that were samples twice on the same day (intra-day variation) and [3] variation in samples taken simultaneously at different sites (upper and lower airways).

Methods

Patients

This prospective study included mechanically ventilated infants at the PICU of the Wilhelmina Children's Hospital with RSV LRTI from November 2007 through February 2009. Wilhelmina Children's Hospital is a tertiary university hospital with a 16 bed PICU facility, and serves as a referral center for the central part of The Netherlands. NPAs and tracheal aspirates (TAs) were taken simultaneously after admission to the PICU. Since viral load is believed to decline over time, follow-up samples were taken 3 times a week to obtain clinical samples with a broad range of viral loads (9;10). When paired sampling of NPAs and TAs was not possible due to insufficient available mucus at either the nose or the trachea, a second attempt to obtain the missing sample was made shortly after the first attempt (usually within 2 hours, never more than 5 hours later). Intra-day variation in load was assessed by taking two samples on the same day (between 8 pm and 4 am), whenever enough mucus was available. All patients provided written informed consent. The local ethics committee approved the study protocol.

Specimens

NPAs and TAs were taken by well-trained nursing staff. Respiratory secretions were aspirated undiluted, directly from nose and tube. Samples (50µL minimum) were transported immediately to the laboratory where they were weighted and diluted with 250µL Dulbecco's Modified Eagle's Medium (DMEM) with 100µg/mL Normocin. To samples that were to be cultured another 300µL was added. Actual dilution of each sample was calculated using sample weight, for later correction of real-time PCR and viral culture results (see below). Samples were processed directly for viral culture and subsequently stored at -80°C for real-time PCR testing.

Quantitative real-time PCR

Nucleic acid preparation

RNA extraction was performed on samples pre-treated with sputolycin (Calbiochem, San Diego, CA, USA) by using a MagnaPure LC total nucleic acid kit (Roche Diagnostics, Mannheim, Germany). Briefly, 200 µL of pretreated sample was mixed with lysis buffer and proteinase K and subsequently incubated with magnetic particles to allow binding of the nucleic acid. Unbound material was removed by several washing steps. The RNA was then eluted in 100 µL of elution buffer and directly used for cDNA synthesis and real-time TaqMan PCR. The isolated viral RNA was reverse transcribed using a MultiScribe reverse transcriptase kit and random hexamers (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's guidelines, followed by RT inactivation for 5 minutes at 95°C. Each 100 µL reaction mixture contained 40 µL of eluted RNA, 10 µL of 10 × RT buffer, 5.5 mmol/L $MgCl_2$, 500 µmol/L of each deoxynucleoside triphosphate, 2.5 µmol/L random hexamer, 0.4 U of RNase inhibitor per microlitre and 1.25 U of Multiscript per µL (all from Applied Biosystems). Murine encephalomyocarditis virus (RNA preparation) was used as internal control.

Real-time PCR

Primers and probes for both RSV A and B were selected using primer express software (PE Applied Biosystems, Foster City, CA, USA) and were based on highly conserved genomic regions of the N gene (11). To cover subgroups, type-specific primers and probes were chosen for RSV A and B. The following forward and reverse primers were used:

RSV A-forward: 5′ AGATCAACTTCTGTCATCCAGCAA
RSV A-reverse: 5′ TTCTGCACATCATAATTAGGAGTATCAAT
RSV B-forward: 5′ AAGATGCAAATCATAAATTCACAGGA
RSV B-reverse: 5′ TGATATCCAGCATCTTTAAGTATCTTTATAGTG
RSV A probe: 5′ CACCATCCAACGGAGCACAGGAGAT
RSV B probe: 5′ TTCCCTTCCTAACCTGGACATAGCATATAACATACCT

Primers and probes were tested for possible interactions to make sure that they could be used together in a multiplex assay. After optimization of primer and probe concentrations, samples were assayed in duplicate in a 50 µL reaction mixture containing 20 µL of cDNA, 25 µL of 2x TaqMan universal PCR master mix (PE Applied Biosystems, Foster City, CA, USA), 900nM concentrations of forward and reverse primers for RSV A, 300 nM concentrations of forward and reverse primers for RSV B, and 60 nM concentrations of each of the probes. The fluorogenic probes 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 with an ABI Prism 7900 sequence detection system under the following conditions: 2 min at 50°C to attain optimal AmpErase uracil-N-glycosylase activity, 10 min at 95°C to

activate the AmpliTaq Gold DNA polymerase, and 45 cycles of 15 s at 95°C and 1 min at 60°C. Samples were controlled for the presence of possible inhibitors of the amplification reaction by the indicated internal control signals of which had to range within clear-cut intervals. For glyceraldehyde 3-phosphate dehydrogenase (GAPDH) RNA detection TaqMan GAPDH Control Reagents kits were used (Applied Biosystems, Foster City, CA, USA). Real-time PCR results were expressed in Ct values. Adjusted Ct values were calculated for positive samples through correction of Ct values for initial dilution of samples, and the adjusted Ct values were used in analyses and figures. Intra-sample variation was assessed by dividing material from 9 NPAs and 9 TAs into three aliquots. Each aliquot was subjected to sputolycin treatment, RNA isolation, cDNA synthesis and amplification separately. Negative samples were set at a Ct value of 45.

Quantitative viral culture

Fresh NPAs were inoculated immediately onto 96-well tissue culture plates to determine the viral titer. For each sample, seven dilution series consisting of 12 five-fold dilutions (30 µL) were added to HEp-2 cell monolayers (70% confluence) in 120 µL DMEM supplemented with 5% fetal calve serum (FCS). RSV quantitative standards were run in parallel with each assay. The RSV quantitative standards were from the supernatant of RSV B grown in HEp-2 cells and frozen at –80°C in 50% FCS. For 10 days, cultures were observed daily for cytopathological effects. The viral titer was calculated as the TCID50 per mL using the Spearman/Kärber method (12).

Statistical analysis

Median values are reported with interquartile ranges (IQRs). Mean values are reported with 95% confidence intervals (CIs). Scatter plots and Bland-Altman plots (13) with 95% limits of agreement were constructed (95% of differences fall between limits of agreement). For negative samples, a Ct value of 45 was used in the analysis. Normalization of RSV Ct values for GAPHD content was calculated as follows: (1) mean GAPDH Ct for a sample set was calculated; (2) deviation from the mean GAPDH Ct was calculated for each sample; (3) the deviation was subtracted from RSV Ct value (i.e. higher loads were assigned to samples with lower GAPDH content). For the comparison of nasopharyngeal and tracheal load two NPAs and two TAs taken on the same day were available in some instances. Ct values of the two NPAs and the two TAs were then averaged to obtain no more than one result-pair per day.

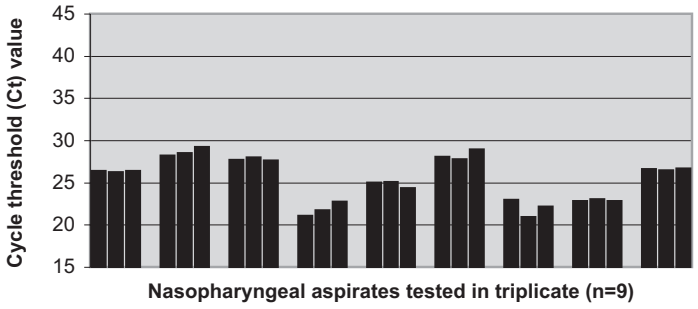
Patients

Thirty-two patients with RSV LRTI were included. Median age (IQR) was 1.6 (1.4) months. Patients remained ventilated for a median (IQR) of 10 (4.0) days.

Variation in the RNA isolation and PCR process (intra-sample variation)

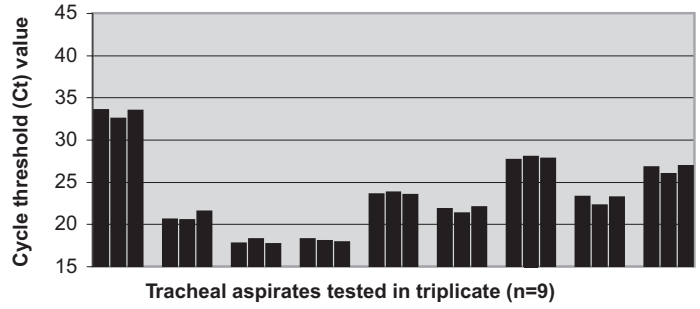
Nine NPAs were divided into three aliquots and RNA isolation and real-time PCR was performed separately for each aliquot. The difference between the 3 aliquots of each NPA was on average 0.74 Ct (range 0.1 to 2.0; Figure 1a). Similarly, 9 TAs were tested showing similar results.. The maximal difference between the three TA-aliquots was on average 0.63 (range 0.3 to 1.1; Figure 1b).

Figure 1a Nasopharyngeal aspirates divided into three aliquots to assess intra-sample variation



The maximal difference between the 3 aliquots of each sample was on average 0.78 Ct (range 0.1 to 2.0).

Figure 1b Tracheal aspirates divided into three aliquots to assess intra-sample variation

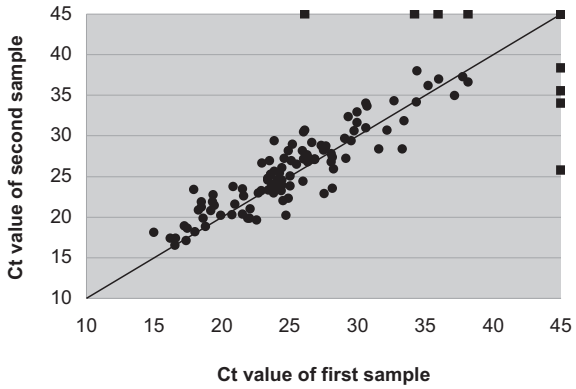


The maximal difference between the 3 aliquots of each sample was on average 0.63 Ct (range 0.3 to 1.1).

Variation in samples taken on the same day (intra-day variation)

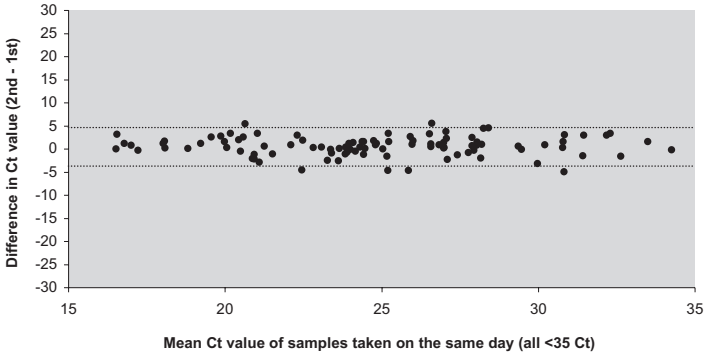
Twenty patients were sampled twice on the same day during their stay at the PICU yielding 138 sample-pairs (77 NPA-pairs and 61 TA-pairs). The mean (SD) time interval between samples was 3.8 (1.9) hours. Hundred-six sample-pairs were both positive and 24 were both negative (Ct=45). Eight sample-pairs consisted of a positive and a negative sample, with Ct values of the positive sample ranging from 25.8 to 38.3 (median 34.9). When results from all sample-pairs were plotted against each other variation was large, mainly due to the eight sample-pairs with one positive and one negative sample (Figure 2). When the double-positive sample-pairs with a mean Ct value of < 35 were analyzed separately, agreement between samples taken on the same day was high, with 95% limits of agreement being -3.6 and 4.8 Ct (Figure 3). Second sample results were on average 0.6 (95% CI 0.2 to 1.0) Ct higher than first sample results, indicating that RSV RNA loads dropped slightly between the first and second sample time-point (p=0.005; paired samples t-test). To determine whether intra-day variability exists due to differences in cellular composition of samples over time, we normalized Ct values of RSV to the content of the reference gene GAPDH. However, normalization for GAPDH did not improve intra-day variability (data not shown). Thus, the difference between the 2 samples over time during the day could not be explained by difference in cellular composition of the aspirates.

Figure 2 Samples taken twice on the same day to assess intra-day variation



Results of 276 samples from 20 patients are shown. The mean (SD) time interval between samples was 3.8 (1.9) hours. Diagonal line represents 100% agreement. Eight sample-pairs were positive in one and negative in the other sample; represented by the squares at the outer limits of the graph (Ct value for negative samples was set at 45). Twenty-four sample-pairs were both negative; represented by the square corresponding to Ct=45 for both samples.

Figure 3 Difference against mean for samples taken twice on the same day with a Ct < 35

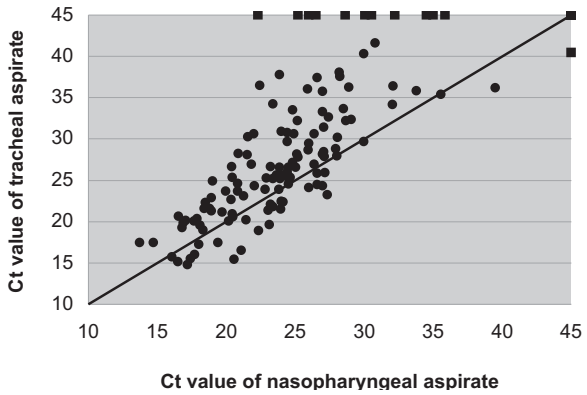


Results of 100 samples from 20 patients (double positive) with a Ct < 35 (high RSV RNA load) are shown. The mean difference between the two time-points is 0.6 Ct (95% CI 0.2 to 1.0). Dotted lines represent 95% limits of agreement (95% of differences lie between -3.6 and 4.8 Ct).

Upper versus lower airway detection

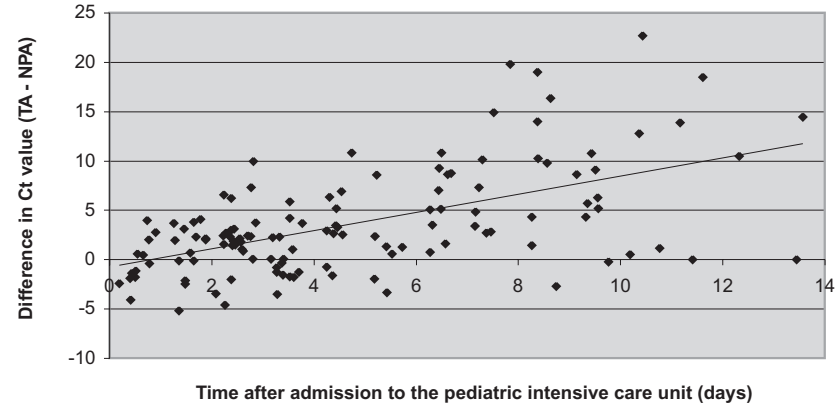
Thirty-one patients provided a total of 138 simultaneously taken, paired NPAs and TAs. Hundred-fifteen sample-pairs were both positive and 13 were both negative (Ct=45). Ten sample-pairs consisted of a positive and a negative sample, with Ct values of the positive sample ranging from 22.3 to 40.5 (median 29.6). In 9 out of these latter 10 sample-pairs the NPA was positive and the TA was negative. When results from all sample-pairs were plotted against each other variation was large, especially towards the higher Ct values (i.e. the lower limit of detection; Figure 4). Notably, sample-pair results towards the higher Ct values were not distributed symmetrically above and under the line of equality. When all samples were analyzed, Ct values of NPA samples were on average 3.3 (95% CI 2.4 to 4.2) Ct lower than TAs (i.e. indicating a higher viral load in NPAs). Subsequently, the difference between NPA and TA RSV RNA load was set out against the number of days after admission (Figure 5). The difference between NPAs and TAs increased during the course of RSV infection ($r=0.52$, $p<0.001$). When only the first sample of each patient was analyzed (n=31) agreement was better, with 95% limits of agreement of -5.2 and 5.8 Ct (Figure 6). In these first samples, NPA Ct values were on average equal to TA Ct values, indicating that in the initial phase of infection NPA load was not higher than TA load.

Figure 4 Simultaneously taken nasopharyngeal and tracheal aspirates



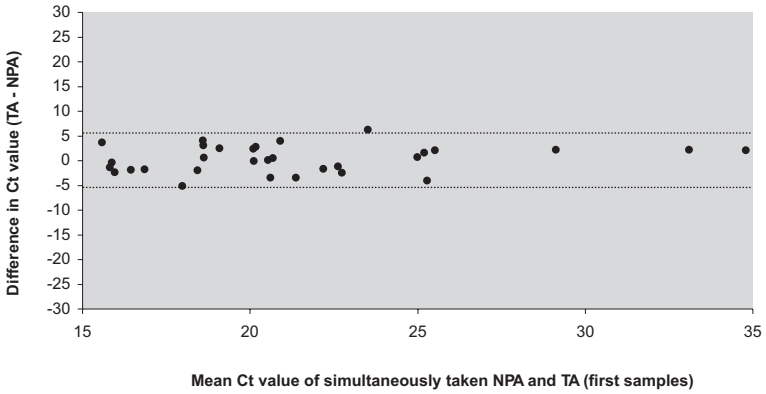
Results of 276 samples from 31 patients are shown. Diagonal line represents 100% agreement. Ten sample-pairs were positive in one and negative in the other sample; represented by the squares at the outer limits of the graph (Ct value for negative samples was set at 45). Thirteen sample-pairs were both negative; represented by the square corresponding to Ct=45 for both samples. Notably, sample-pair results towards the higher Ct values were not distributed symmetrically above and under the line of equality. Ct values of NPA samples were on average 3.3 (95% CI 2.4 to 4.2) Ct lower than TAs.

Figure 5 Difference between simultaneously taken nasopharyngeal and tracheal aspirates during the course of RSV infection



Results of 266 samples from 31 patients are shown (results of the first 14 days after admission to the pediatric intensive care unit). During the course of infection, nasal Ct values gradually remained lower than tracheal Ct value, indicating a higher viral load in the nose. Dotted line represents linear correlation (r=0.52, p<0.001). Abbreviations: NPA: nasopharyngeal aspirate; TA: tracheal aspirate.

Figure 6 Difference against mean for simultaneously taken nasopharyngeal and tracheal aspirates

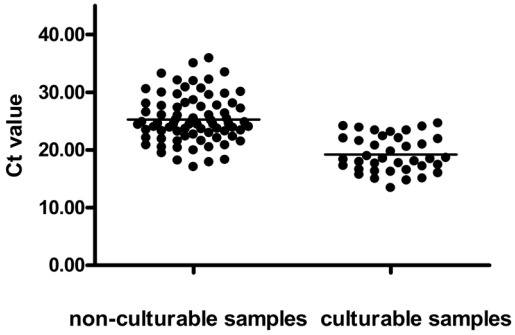


Results of 31 samples from 31 patients are shown. The first nasopharyngeal and tracheal aspirate sample-pairs at admission at the PICU for each patient are shown. The mean difference is not significantly different from zero. Dotted lines represent 95% limits of agreement (95% of differences lie between -5.2 and 5.8 Ct). Abbreviations: TA: tracheal aspirate; NPA: nasopharyngeal aspirate.

Molecular RNA detection compared to viral culture

Viral culture had a lower sensitivity than real-time PCR, detecting RSV in only 20 out of 32 included RSV-PCR positive patients (63%). Samples with a positive viral culture had lower Ct values than samples with a negative culture (p=<0.001; Mann-Whitney U test; Figure 7).

Figure 7 Real-time PCR results of samples with positive and negative viral cultures



Results of 115 samples from 32 patients are shown. Median (IQR) Ct value of culture negative samples and positive samples were 24.5 (5.4) and 18.5 (5.4), respectively (p<0.001; Mann-Whitney U test).

Discussion

The present study aims to validate the use of real-time PCR for quantification of RSV RNA load in respiratory secretions by assessing the different steps of quantitative detection that may introduce variability in results. First, we showed that intra-sample variation is minimal (maximal variability 2.0 Ct). Second, we found that intra-day variation in RSV RNA load was low in samples with high viral loads (95% limits of agreement were -3.6 to 4.8 for samples <35 Ct). Third, we showed that nasal loads were comparable to tracheal loads during the initial phase of infection (95% limits of agreement were -5.2 to +5.8 Ct for first samples from each patient). Overall, this indicates that differences in lower airway RSV RNA load of approximately 5 Ct or more, can reliably be detected taking NPAs during the initial phase of RSV infection.

Two limitations of our study deserve further discussion. First, this was a clinical study performed at the PICU. Although samples were taken by well-trained nursing staff and immediately transported to the laboratory by a dedicated investigator, some personal variation in sampling procedures cannot be fully excluded. On the other hand, because of this clinical study design, our results do accurately reflect the reliability of real-time PCR that can be expected when quantification is performed in daily clinical practice. Second, it is not certain that RSV RNA load is representative of the concentration of infectious virus. This uncertainty is reflected in the fact that only 20 out of 32 included RSV-PCR positive patients had a positive viral culture. The low sensitivity of viral culture is a general problem recognized in previous studies (14;15). It is unlikely that our PCR results reflect the presence of non-infectious viral RNA sequences since patients were repeatedly sampled during the course of infection and RSV RNA load was found to be consistently high over several days up to two weeks (data not shown), whereas non-replicating viral RNA sequences would have been cleared within hours. Thus, the low sensitivity of culture underscores the need for adequate validation of real-time PCR as a means of quantitative detection.

Our data regarding the agreement between nasal and tracheal viral load in the initial phase of infection, confirm the results from Wright and colleagues (16), who used plaque assays to assess viral load, and Perkins (17) who used real-time PCR. These authors reported an even better agreement between NPAs and TAs. This is likely to be explained by the fact that they evaluated only patients with positive viral cultures, most likely representing patients with higher viral loads. Our observation that NPAs show a higher RSV load than TAs in later phases of infection needs to be confirmed by future studies. The pathophysiologic mechanisms by which this difference may be caused should be the focus of further investigations, e.g. improved viral clearance in the lower respiratory tract.

Overall, we suggest that mucus samples can be tested reliably with real-time PCR to quantify RSV RNA load, to identify differences in load in the order of magnitude of 5 Ct or more. This order of magnitude correlates to what is expected to be clinically relevant in RSV respiratory infections, since inoculation of patients with RSV leads to exponential growth curves of viral load with loads rising from around 10 to 1000 TCID50 (≈ 7 Ct rise) in a matter of hours (18). Similarly, small differences (0 to 5 Ct) are probably irrelevant, and real-time PCR is not a useful tool to detect differences in this order of magnitude due to [1] intra-sample variation (max. 2 Ct), [2] intra-day variation (max. 5 Ct), and [3] NPA/TA variation (max. 6 Ct). Furthermore, caution should be taken into account in the interpretation of Ct values of 35 and higher, since at this concentration capturing RNA in clinical samples becomes a stochastic process. As part of another study we analyzed the course of RSV load during infection and found that RSV load was highest upon admission (unpublished data). Since both intra-day variation and NPA/TA variation are smallest in samples with high viral loads taken shortly after admission, it is advisable to sample patients as early in the course of infection as possible.

In conclusion, quantitative real-time PCR detection of RSV RNA in undiluted mucus is a reliable method to quantify viral loads in patients with LRTI. Nasal samples in the initial phase of infection can be used accurately to predict RSV RNA loads in the lower airways.

Acknowledgments

B.P. Waalewijn, MD, and E.S. Schouten, MD, are kindly acknowledged for their help with the sample collection. This study was supported by an “Alexandre Suerman program” MD/PhD grant to A.C. van de Pol, MD, from the University Medical Center Utrecht, The Netherlands.

Reference List

(1) Hall CB, Weinberg GA, Iwane MK, Blumkin AK, Edwards KM, Staat MA, Auinger P, Griffin MR, Poehling KA, Erdman D, Grijalva CG, Zhu Y, Szilagyi P. The burden of respiratory syncytial virus infection in young children. N Engl J Med 2009 February 5;360(6):588-98.

(2) van de Pol AC, Wolfs TF, Jansen NJ, van Loon AM, Rossen JW. Diagnostic value of real-time polymerase chain reaction to detect viruses in young children admitted to the paediatric intensive care unit with lower respiratory tract infection. Crit Care 2006;10(2):R61.

(3) Hall CB, Douglas RG, Jr., Geiman JM. Respiratory syncytial virus infections in infants: quantitation and duration of shedding. J Pediatr 1976 July;89(1):11-5.
(4) Henrickson KJ, Hall CB. Diagnostic assays for respiratory syncytial virus disease. Pediatr Infect Dis J 2007 November;26(11 Suppl):S36-S40.
(5) Kuypers J, Wright N, Ferrenberg J, Huang ML, Cent A, Corey L, Morrow R. Comparison of real-time PCR assays with fluorescent-antibody assays for diagnosis of respiratory virus infections in children. J Clin Microbiol 2006 July;44(7):2382-8.
(6) Perkins SM, Webb DL, Torrance SA, El SC, Harrison LM, Aitken JA, Patel A, DeVincenzo JP. Comparison of a real-time reverse transcriptase PCR assay and a culture technique for quantitative assessment of viral load in children naturally infected with respiratory syncytial virus. J Clin Microbiol 2005 May;43(5):2356-62.
(7) van de Pol AC, van Loon AM, Wolfs TF, Jansen NJ, Nijhuis M, Breteler EK, Schuurman R, Rossen JW. Increased detection of respiratory syncytial virus, influenza viruses, parainfluenza viruses, and adenoviruses with real-time PCR in samples from patients with respiratory symptoms. J Clin Microbiol 2007 July;45(7):2260-2.
(8) Falsey AR, Formica MA, Treanor JJ, Walsh EE. Comparison of quantitative reverse transcription-PCR to viral culture for assessment of respiratory syncytial virus shedding. J Clin Microbiol 2003 September;41(9):4160-5.
(9) van Elden LJ, Sachs AP, van Loon AM, Haarman M, van d, V, Kimman TG, Zuithoff P, Schipper PJ, Verheij TJ, Nijhuis M. Enhanced severity of virus associated lower respiratory tract disease in asthma patients may not be associated with delayed viral clearance and increased viral load in the upper respiratory tract. J Clin Virol 2008 February;41(2):116-21.
(10) van Woensel JB, Lutter R, Biezeveld MH, Dekker T, Nijhuis M, van Aalderen WM, Kuijpers TW. Effect of dexamethasone on tracheal viral load and interleukin-8 tracheal concentration in children with respiratory syncytial virus infection. Pediatr Infect Dis J 2003 August;22(8):721-6.
(11) van de Pol AC, van Loon AM, Wolfs TF, Jansen NJ, Nijhuis M, Breteler EK, Schuurman R, Rossen JW. Increased detection of respiratory syncytial virus, influenza viruses, parainfluenza viruses, and adenoviruses with real-time PCR in samples from patients with respiratory symptoms. J Clin Microbiol 2007 July;45(7):2260-2.
(12) Hierholzer JC, Killington RA. Virus isolation and quantitation. In: Mahy BW, Kagro H.O., editors. Virology methods manual.United Kingdom: Academic press limited; 1996. p. 25-46.
(13) Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet 1986 February 8;1(8476):307-10.
(14) Hall CB, Douglas RG, Jr., Geiman JM. Respiratory syncytial virus infections in infants: quantitation and duration of shedding. J Pediatr 1976 July;89(1):11-5.
(15) Henrickson KJ, Hall CB. Diagnostic assays for respiratory syncytial virus disease. Pediatr Infect Dis J 2007 November;26(11 Suppl):S36-S40.

(16) Wright PF, Gruber WC, Peters M, Reed G, Zhu Y, Robinson F, Coleman-Dockery S, Graham BS. Illness severity, viral shedding, and antibody responses in infants hospitalized with bronchiolitis caused by respiratory syncytial virus. J Infect Dis 2002 April 15;185(8):1011-8.
(17) Perkins SM, Webb DL, Torrance SA, El SC, Harrison LM, Aitken JA, Patel A, DeVincenzo JP. Comparison of a real-time reverse transcriptase PCR assay and a culture technique for quantitative assessment of viral load in children naturally infected with respiratory syncytial virus. J Clin Microbiol 2005 May;43(5):2356-62.
(18) Falsey AR, Formica MA, Treanor JJ, Walsh EE. Comparison of quantitative reverse transcription-PCR to viral culture for assessment of respiratory syncytial virus shedding. J Clin Microbiol 2003 September;41(9):4160-5.

CHAPTER 9

A systemic neutrophil response precedes robust CD8+ T cell activation during natural Respiratory Syncytial Virus infection in infants

Michaël V. Lukens*
Alma C. van de Pol*
Frank E.J. Coenjaerts
Nicolaas J.G. Jansen
Vera M. Kamp
Jan L.L. Kimpen
John W.A. Rossen
Laurien H. Ulfman
Carline E.A. Tacke
Marco C. Viveen
Leo Koenderman
Tom F.W. Wolfs
Grada M. van Bleek

**These authors contributed equally to this paper*
Provisionally accepted by Journal of Virology

Abstract

Severe primary Respiratory Syncytial Virus (RSV) infections are characterized by bronchiolitis accompanied by wheezing. Controversy exists whether infants suffer from virus induced lung pathology or from excessive immune responses. Furthermore, detailed knowledge on the development of primary T cell responses to viral infections in infants is lacking. We studied the dynamics of innate neutrophil and adaptive T cell responses in peripheral blood in relation to viral load and parameters of disease in infants admitted to the intensive care unit with severe RSV infection. Analysis of primary T cell responses showed a substantial $CD8^+$ T cell activation which peaked during convalescence. A strong neutrophil response, characterized by mobilization of bone marrow derived neutrophil precursors preceded the peak in T cell activation. The kinetics of this neutrophil response followed the peak of clinical symptoms and viral load with a 2-3 day delay. From the sequence of events we conclude that $CD8^+$ T cells responses, initiated during primary RSV infections, are unlikely to contribute to disease when it is most severe. The mobilization of precursor neutrophils might reflect the strong neutrophil influx into the airways, which is a characteristic feature during RSV infections and might be an integral pathogenic process in this disease.

Introduction

Viral infections are characterized by a dynamic interplay between the pathogen and defensive innate and adaptive immune responses of the host (1;2). Upon infection, virus specific structural components are recognized by pattern recognition receptors of the host, which triggers a mechanism aimed at the suppression of virus replication and eventually virus elimination. Each virus has a characteristic signature of triggering innate immune receptors and methods to counteract immune responses of the host, which ultimately results in an immune response tailored to the particular properties of the infecting virus (3).

Most insights in the sequence of events occurring during viral infections have been obtained from animal experiments, where the immunological control of viral infections can be studied in detail. In many murine models the crucial role of $CD8^+$ T cells in complete elimination of the virus during acute infections has been well established (4-6). However, both viral-induced damage and immune pathology might contribute to the disease depending on the type of viral infection and/or the intensity of innate and adaptive immune responses triggered (6-11).

Primary infections with Respiratory Syncytial Virus (RSV) can cause severe bronchiolitis and pneumonia in infants (12). For RSV the mouse is not a good model to study primary disease because the virus replicates poorly in murine cells. Hence to obtain insight in the mechanism of disease caused by RSV infection studies in humans or non human primate models are needed. We and others have shown that RSV infection causes a strong influx of neutrophils into the airways (13-15). In addition, we have recently shown that substantial virus specific $CD8^+$ T cell responses can be elicited in infants with severe RSV infections (15). However, it is still a controversial issue whether the severe manifestations of lower respiratory tract disease are caused directly by the virus or by innate and/or adaptive immune responses triggered by RSV (6;16-18). In our previous work we found no relation between the severity of disease and the number of virus specific $CD8^+$ T cells in peripheral blood (15). Moreover, a direct role of viral load or different viral strains on disease severity has neither been established convincingly (19;20).

Data on the development of primary T cell responses in infants (< 6 months old) during acute viral infections and after vaccinations is sparse. It is generally accepted that the infant immune system is immature and less effective compared to older children or adults. This has been shown by lower activation and/or Th2 polarized adaptive immune responses (21-23). For RSV-induced disease it has been suggested that a Th2 biased immune response might be correlated with disease (24-26), but this idea has been challenged by others (27-29).

Currently, there is no RSV vaccine and the only preventive treatment available is a humanized neutralizing antibody specific for the Fusion protein of RSV which is administered to high risk groups and is effective in about 60 % of children (30). Immune suppressive or antiviral treatments during severe RSV disease have marginal to no effect (31-33). Insights in the kinetics of viral load and disease course in relation to activation of the innate and adaptive immune response will shed light on factors that attribute to severe RSV-induced disease and possibly provide leads for the development of curative treatment. We therefore monitored the dynamics of these parameters in infants admitted to the pediatric intensive care unit (ICU) with severe primary RSV infections. During primary RSV infection peak values of viral load and disease severity were followed by the exhaustion of the peripheral blood neutrophil pool, indicating a strong innate immune response closely associated with the peak of disease. We further showed that this natural respiratory infection elicited a strong primary $CD8^+$ T cell response in the very young patients (< 3 months). This T cell response was undetectable at the moment of hospitalization when infants were severely ill, and peaked at convalescence. Therefore, severe primary RSV disease does not seem to be caused by inadequate or exaggerated T cell responses but is most likely initiated by viral damage followed by intense innate immune processes.

Methods

Study population, clinical characteristics and sample collection

The study population consisted of 22 infants younger than 52 weeks of age, admitted to the pediatric ICU of the Wilhelmina Children's Hospital during the winter seasons of 2007-2009. All patients required mechanical ventilation because of respiratory failure due to RSV lower respiratory tract infection. Children with immune deficiencies were not included in the study. To measure the development of T cell and neutrophil responses in time, 1 ml of blood was collected in EDTA tubes directly after written parental informed consent and every other day until discharge from the ICU. At the same time intervals, undiluted nasopharyngeal aspirates (NPA) and tracheal aspirates (TA) were collected for the determination of respiratory viruses. To measure the course of disease severity, the ventilation index was calculated as the product of partial pressure of CO_2 (mm Hg) x peak airway pressure (cm H_2O) x respiratory rate (breaths per min) divided by 1000 (34). In patients on the high frequency oscillation ventilator (i.e. no respiratory rate available; n=2) the oxygenation index was used, calculated as the inspired oxygen fraction x mean airway pressure (cm H_2O) divided partial pressure of oxygen in arterial blood. The control group consisted of 7 healthy infants around 4 weeks of age without current or recent clinical symptoms of a respiratory infection. Parents of patients and controls gave their informed written consent. The study was approved by the Medical Ethical Committee of the University Medical Center, Utrecht, the Netherlands.

Respiratory virus detection and quantification

The presence of RSV and other respiratory viruses was determined by real-time (reverse transcriptase) PCR on NPA and TA. Nucleic acid was isolated as previously described (35;36). Shortly, nucleic acids were extracted using the MagnaPure LC total nucleic acid kit (Roche Diagnostics, Mannheim, Germany) and eluted in 100 µl elution buffer. cDNA was synthesized by using MultiScribe reverse transcriptase (RT) and random hexamers (both from Applied Biosystems, Foster City, CA). Each 100 µl reaction mixture contained 40 µl of the eluted RNA. After incubation for 10 minutes at 25 °C, RT was carried out for 30 min at 48 °C, followed by RT inactivation for 5 min at 95 °C.

Detection of respiratory viruses was performed in parallel, using real-time PCR assays specific for: RSV A and B, influenza virus A and B, parainfluenza virus 1 to 4, human rhinovirus, adenovirus, human corona virus OC43, NL63 and 229E, human metapneumovirus, and human bocavirus. Real-time PCR procedures were performed as previously described (35;36).

Briefly, samples were assayed in a 50 µl reaction mixture containing 20 µl of cDNA, 25 µl of 2x TaqMan universal PCR master mix (PE Applied Biosystems, Foster City, CA), and 200-900 nM concentrations of forward and reverse primers and 60-200 nM concentrations of each of the probes. Amplification and detection were performed with an ABI Prism 7900 sequence detection system. Efficient extraction and amplification was monitored through the internal control viruses (RNA virus; murine encephalomyocarditis virus and DNA virus; phocine herpes virus) (37). Real-time PCR results were expressed in semi-quantitative Ct values. For RSV, Ct values were converted to number of particles/ml using standardization curves generated with EM counted stocks. The amount of infectious RSV particles present in fresh NPA was determined by an end-point dilution assay. Hep-2 cells grown in 96-well plates were inoculated with serial dilutions of the NPA in culture medium. RSV quantitative standards were run in parallel with each assay. For 10 days, cultures were observed daily for cytopathological effects. The 50 % culture infectious dose (TCID50) values were determined using the Spearman/Kärber relationship (38).

Phenotyping of immune cells

Whole blood was stained with different extra-cellular mAbs for 30 min on ice. The following mAbs were used to phenotype $CD8^+$ T cell populations; FITC-conjugated anti-CD8 (CLB-T8/4, 4H8), -CD45RO (UCHL1), -CCR5 (2D7), PE-conjugated anti-CD3 (SK7), -CD8 (SK1), -CD127 (R34.34), -CCR7 (3D12), PercP-conjugated anti-CD8 (SK1), and APC-conjugated anti-CD3 (SK7), -CD27 (L128) were all purchased from BD Biosciences (San Jose, CA) except CD8-FITC (Sanquin, Amsterdam, The Netherlands) and CD127-PE (Immunotech, Marseille, France). For intracellular staining cells were permeabilized and fixed using FACS permeabilizing/fixation solution (Perm/Fix, BD Biosciences). Cells were stained intra-cellular with FITC-conjugated anti-Ki-67 (B56), anti-Perforin (δG9), ALEXA647-conjugated anti-Granzyme B (GB11) or their isotype controls (BD Biosciences).

The following mAbs were used for the staining of neutrophils and eosinophils. PE-conjugated anti-CD49d (9F10) and ALEXA647-conjugated anti-CD16 (3G8) were purchased from BD Biosciences. After cell surface staining, erythrocytes were lysed using lysis buffer containing 155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM Na_2EDTA for 15 min. Cells were washed twice in FACS buffer and analyzed selected on FSC/SSC and marker expression patterns by FACS Calibur flow cytometer and CellQuest software or sorted on a FACS Aria (BD Biosciences).

Statistical analysis

Data was normalized whereby the value of 100 % was assigned to the largest number in a patient data set and 0 % to the smallest number in the same data set (GraphPad 4.0, Prism). The Pearson's correlation coefficients were calculated for (1) peak viral load (trachea), (2) peak percentage of $CD16^{int}$ neutrophils, and (3) peak percentage of $CCR5^{+}CCR7^{-}CD8^{+}$ T cells with (a) length of stay (LoS) at the ICU and (b) peak ventilation index on day 0-2 (SPSS 15.0 for Windows).

Results

Study setup and clinical characteristics of patients

Previously, we have demonstrated that during severe primary RSV infections, virus specific T cell responses could be detected in peripheral blood and bronchial alveolar lavage of infants with severe RSV bronchiolitis (15). We initiated the present study to elaborate on our previous work and gain more insight into virus clearance, disease severity and immune responses by studying the kinetics of neutrophil and developing T cell and responses in relation to viral load, and parameters of disease severity.

Twenty two patients <52 weeks of age were included in the study. They all had severe RSV bronchiolitis and required mechanical ventilation due to respiratory failure (for demographics see Table 1). During the study five patients were excluded based on bacterial superinfection with positive bacterial cultures. Of the 17 patients who were included in the final analysis, 16 were < 3 months of age and one child was 5 months old. Peripheral blood samples, nasopharyngeal aspirates (NPA) and tracheal aspirates (TA) were taken at the same time intervals.
Upon study inclusion parental reported onset of symptoms (rhinitis, cough, fever, wheezing, apneas, dyspnea, vomiting, and diarrhea), gestational age at birth, and birth weight were recorded. On average the RSV patients were symptomatic for 5.4 (SEM 0.7) days before admission to the ICU. Severity of illness was assessed using (i) Length of Stay (LoS) at the ICU (days), and (ii) parameters of mechanical ventilation (Figure 1). Since the exact moment of infection was unknown, the day of first clinical symptoms of a respiratory infection as reported by the parents was regarded as day zero. The disease was most severe in the first 2-3 days on the ICU in all patients.

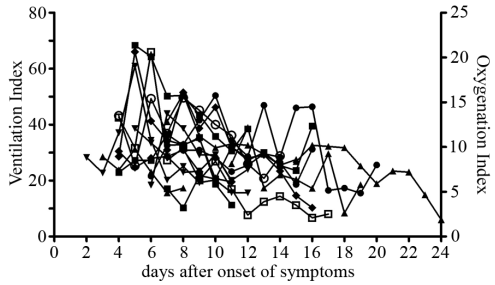
Table 1 Clinical characteristics of patients and healthy controls

	RSV PATIENTS (N=17)	HEALTHY CONTROLS (N=7)
Male/female, no.	11/6	3/4
Age, w, mean (SEM)	7.2 (1.2)	5.6 (0.4)
Premature birth <36w, no. (%)	3 (18)	0 (0)
Symptom onset*, d, mean (SEM)	5.4 (0.7)	NA
Viral culture positive for RSV**, no. (%)	11 (65)	NA
Viral co-infection, no. (%)	5 (31)a	NA
Bacterial culture positive ≤2d, no. (%)	12 (71)b	NA
Duration of ventilation, d, mean (SEM)	10.4 (0.6)	NA
Length of stay, d, mean (SEM)	11.3 (0.6)	NA

[a] *Human rhinovirus (n=5) and human coronavirus (n=1).* [b] ***Haemophilus influenza** (n=7), **Staphylococcus aureus** (n=5), and **Moraxella catarrhalis** (n=4) were most commonly found.*

** before admittance to the ICU. ** all patients were positive by RSV PCR. W, weeks; d, days; NA, not applicable.*

Figure 1 Kinetics of parameters of mechanical ventilation

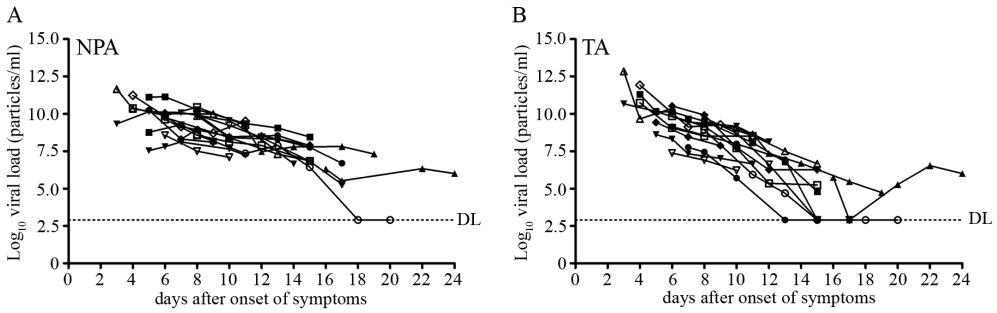


Day 0 denotes the first reported symptomatic day. Disease severity of RSV patients, based on oxygenation index (OI), open symbols, right x-as or ventilation index (VI), closed symbols left x-axis, determined during the stay at the ICU.

Kinetics of RSV load

Viral RNA loads were measured in NPA and TA by real-time PCR and quantitative viral culture. Viral RNA load was maximal in the first samples in both NPA and TA (Figure 2A and B), decreased during the hospital stay, but could still be detected in most patients in the NPA at the time of discharge (around day 17 after onset of symptoms; Figure 2A). Interestingly, RSV RNA was cleared more rapidly in the lower than in the upper airways (Figure 2B versus 2A). In only 65 % of the patients we were able to detect infectious virus particles by viral culture, and virus could never be cultured beyond day 10 after onset of symptoms. This was well before discharge from the ICU in the majority of patients (data not shown).

Figure 2 Decrease in viral load



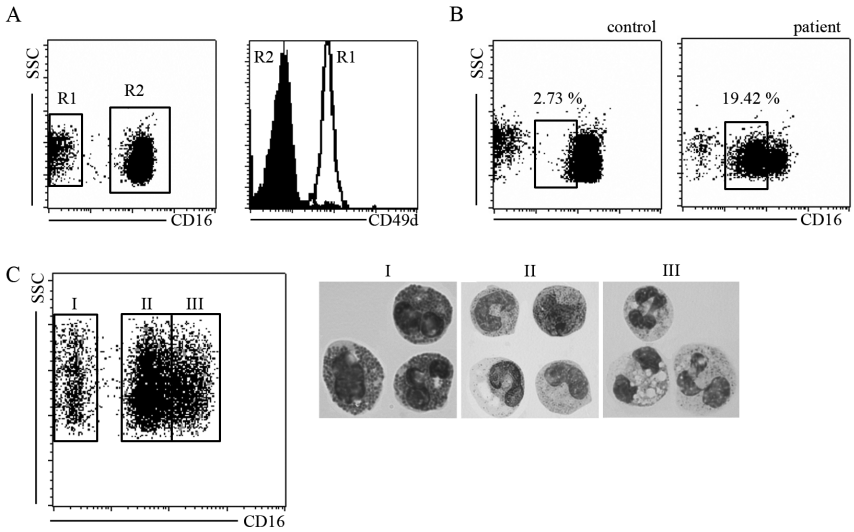
RSV load was determined by PCR in NPA (A) or TA (B) during the stay at the ICU, and plotted as log_{10} viral particles/ml. The lower limit of detection (DL) of the PCR was 2.9 log_{10} viral particles/ml RSV.

Recruitment of neutrophil precursors into the blood during RSV infection

RSV infection is characterized by a massive influx of mainly neutrophils into the airways compared to control patients undergoing surgery for non respiratory tract related disease (13-15). Neutrophils were phenotyped in order to monitor systemic effects involved in neutrophil recruitment into airways of RSV patients. Neutrophils were identified based on FSC/SSC patterns and staining with anti-FcγRIII (CD16) and VLA-4 (CD49d) ($CD16^{hi}CD49d^{neg}$) and could easily be separated from eosinophils ($CD16^{neg}CD49d^{hi}$, Figure 3A). Interestingly, during the course of RSV infection we observed the appearance of a population of neutrophils with an intermediate CD16 expression (Figure 3B). Banded neutrophils or their precursors recruited from the bone marrow into the blood by G-SCF or inflammatory conditions in general have a lower expression of CD16 (39;40). To confirm that this was also the case during RSV infections and not due to prolonged stimulation, we sorted granulocytes in three different populations based on negative, intermediate and high CD16

expression (41). Cytospins of these sorted cell populations were performed and the presence of eosinophils ($CD16^{neg}$), and mature/activated neutrophils ($CD16^{hi}$) were identified by May-Günwald Giemsa staining (Figure 3C). The population of cells characterized by intermediate expression of CD16 ($CD16^{int}$) was a heterogeneous population of neutrophil precursors consisting of myelocytes and metamyelocytes and banded neutrophils, which are neutrophil precursors normally residing in the bone marrow (Figure 3C) (42;43). The same cell types were also observed in direct bloodfilms of RSV patients (data not shown).

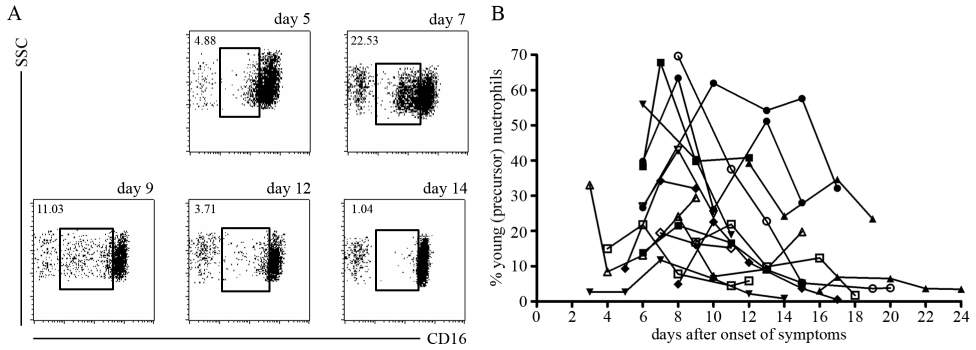
Figure 3 Identification of blood neutrophils



(A) Whole blood of a healthy control was stained with anti-CD16, and -CD49d. Granulocytes were identified based on FSC/SSC patterns and CD16 staining (left panel), neutrophils were identified based on low expression of CD49d, while eosinophils had a high expression of CD49d (right panel). (B) Expression pattern of CD16 on granulocytes from a healthy control (left panel) or RSV patient (right panel). (C) Gating strategies (FACS plot) for sort of eosinophils and neutrophils of an RSV patient, (right panels) cytospin with May-Günwald Giemsa staining of sorted eosinophils, neutrophil precursors and mature neutrophils.

We quantified the percentage of $CD16^{int}CD49d^{neg}$ neutrophil precursors within the total granulocyte gate (neutrophils and eosinophils) in time during the stay at the ICU.
We found very low numbers of neutrophil precursors in the blood of RSV patients at admission to the ICU, but they appeared a few days after admission to the ICU (Figure 4A).
The main increase in neutrophil precursors for all of the RSV patients was between day 7 and 9 after the onset of symptoms (Figure 4B) and subsided a few days later.

Figure 4 Influx of neutrophil precursors into peripheral blood



(A) Whole blood of a RSV patient was stained with anti-CD16, and the percentage of precursor neutrophils within the granulocyte gate was enumerated. (B) Kinetics of neutrophil precursor (CD16^{int} expression) recruitment into the blood.

Identification of activated CD8$^+$ T cells in peripheral blood

Because in infants younger than 6 months of age the majority of T cells are still naïve, the presence of a RSV specific T cell response could be readily identified by a large set of phenotypic markers that are known to be expressed on activated and/or memory CD8$^+$ T cells (15).
To monitor CD8$^+$ T cell kinetics we stained whole blood samples with different combinations of activation markers. First, we used the combination of antibodies recognizing chemokine receptors CCR5 and CCR7. CCR7 is expressed on naïve and central memory T cells but not on effector T cells. CCR7 is the receptor for the ligands CCL19 and CCL21 that are mainly expressed in lymphoid organs (44). The down-regulation of CCR7 allows T cells to leave lymphoid organs while upregulation of CCR5 directs the migration to inflamed sites where the ligand RANTES (CCL5) is expressed as in the RSV infected lung (45;46). Figure 5A shows an example of the presence of activated CCR7$^-$CCR5$^+$ effector CD8$^+$ T cells (31.6 % of CD8$^+$ T cells) in a patient during severe RSV bronchiolitis compared with a typical example of a healthy age matched control child (0.9 % of CD8$^+$ T cells).

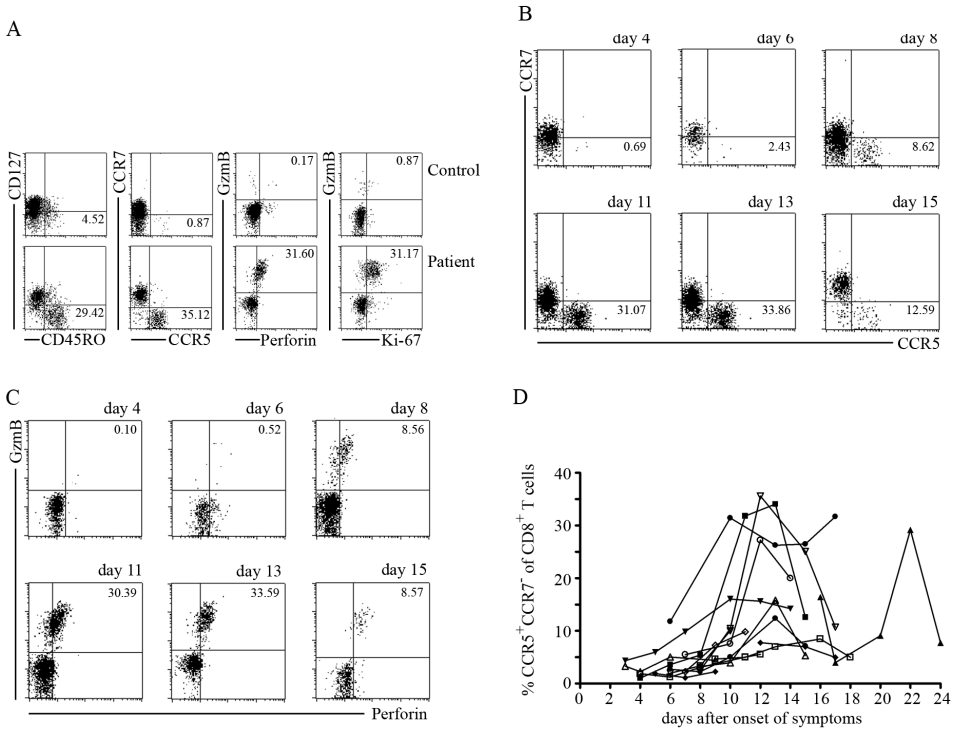
A second way to identify effector CD8$^+$ T cells in this young age group is the combination of CD127/CD45RO staining. CD127 is the receptor for IL-7 which is expressed on naïve and memory T cells and is required for homeostatic proliferation (47). The IL-7 receptor is down-regulated on effector T cells that are also identified by upregulated expression of CD45RO. Figure 5A shows that the combination of these markers identifies a similar fraction of effector T cells in the RSV patient which is enhanced compared to the control (respectively 29.4% versus 4.5% of CD8$^+$ T cells). The combination staining of Granzyme B and perforin identifies CD8$^+$ T cells with lytic

potential, while Ki-67 stains proliferating or recently divided cells (48-50). Figure 5A shows that in the same patient also these combinations of markers identified a similar fraction of $CD8^+$ T cells, GzmB/perforin (33.6 % versus 0.2 % in control) or GzmB/Ki67 (31.2 % versus 0.9 % in control). In previous work we have shown that these markers are all co-expressed on recently activated cells, that are for a substantial part also virus specific (15) and unpublished results). Figure 5B and 5C show an example of the developing $CD8^+$ T cell response in time in a second RSV patient using the combination staining for CCR7/CCR5 (Figure 5B) and GzmB/perforin (Figure 5C). These data illustrate that the kinetics of the $CD8^+$ T cell response can be visualized by both combinations of markers.

The identification of $CCR5^+CCR7^-$ and $GzmB^+Perforin^+$ effector $CD8^+$ T cells in peripheral blood, allowed us to track the kinetics of the $CD8^+$ T cell response in patients during the hospital stay. In Figure 5D, the kinetics of the $CD8^+$ T cell response is summarized for the patient group, based on expression of $CCR5^+/CCR7^-$. The data represent the percentage of effector $CD8^+$ T cells found on the depicted days, whereby day 0 was the day of first clinical symptoms. While the peak level of the T cell response varied significantly between patients (varying from 5 % to 35 %), the kinetics of the response was similar in all patients, peaking between day 11 and 15 after the onset of symptoms (Figure 5D). In one patient the peak of the T cell response was found 22 days after the onset of symptoms. This child had symptoms of common cold for two weeks before admission to the hospital. Although we did not detect a different virus in this patient, it remains possible that a different viral infection preceded the RSV infection.

On admission, when viral load and disease severity peaked, the percentage of effector $CD8^+$ T cells was extremely low and comparable to the percentages observed in healthy control children (1.5 % SEM 0.2). After extensive expansion the number of effector $CD8^+$ T cells in blood of RSV patients dropped from peak levels by the time of discharge. The kinetics and peak values in the effector $CD8^+$T cell response as measured by chemokine markers ($CCR5^+CCR7^-$ $CD8^+$ T cells) was very similar to observations made with $GzmB^+Perforin^+$ stained $CD8^+$ T cells (data not shown).

Figure 5 Identification of activated CD8⁺ T cells in blood



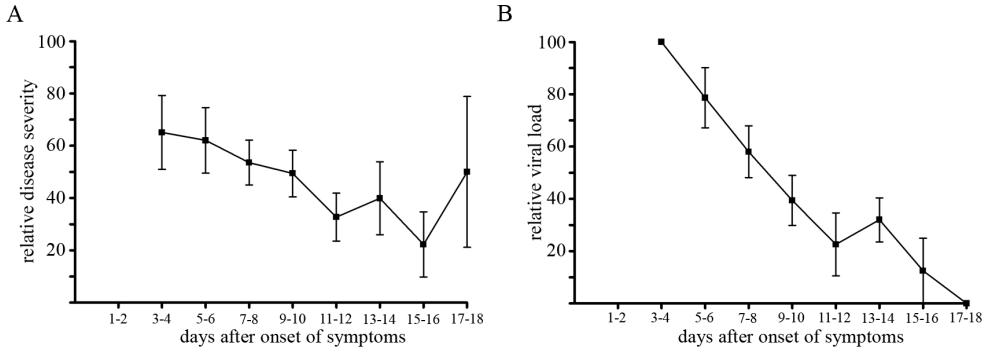
(A) Whole blood of a healthy control infant (upper panel) and a 12 week old RSV patient (lower panel) drawn at day 11 after onset of symptoms, was surface stained with anti-CD3, -CD8, -CD45RO, -CD127, -CCR5, -CCR7 or stained intracellular with anti-GzmB, -Perforin and -Ki-67. CD8⁺ T cells were identified based of FSC/SSC lymphocyte gating and then on the expression of CD3⁺CD8⁺. (B) Whole blood of a second (2 week old) RSV patient was drawn at admission and every other day until discharge. The blood was surface stained with (B) anti-CD3, -CD8, -CCR5, -CCR7 or (C) stained with anti-CD3, -CD8, and intracellular with -GzmB and -Perforin. The percentages of activated CD8⁺ T cells are given in the respective quadrant. The experiments shown are representative examples of individual (13) RSV patients and (7) healthy controls. In all patients and controls a similar pattern of activation markers and chemokine receptor expression was observed, however the level of activation differed between patients. (D) The kinetics of CD8⁺ T cell activation was measured in whole blood during the stay at the ICU based on the expression pattern of CCR5 and CCR7 on CD3⁺CD8⁺ lymphocytes. Due to technical reasons, we could only measure T cell activation in 13 out of the 17 patients.

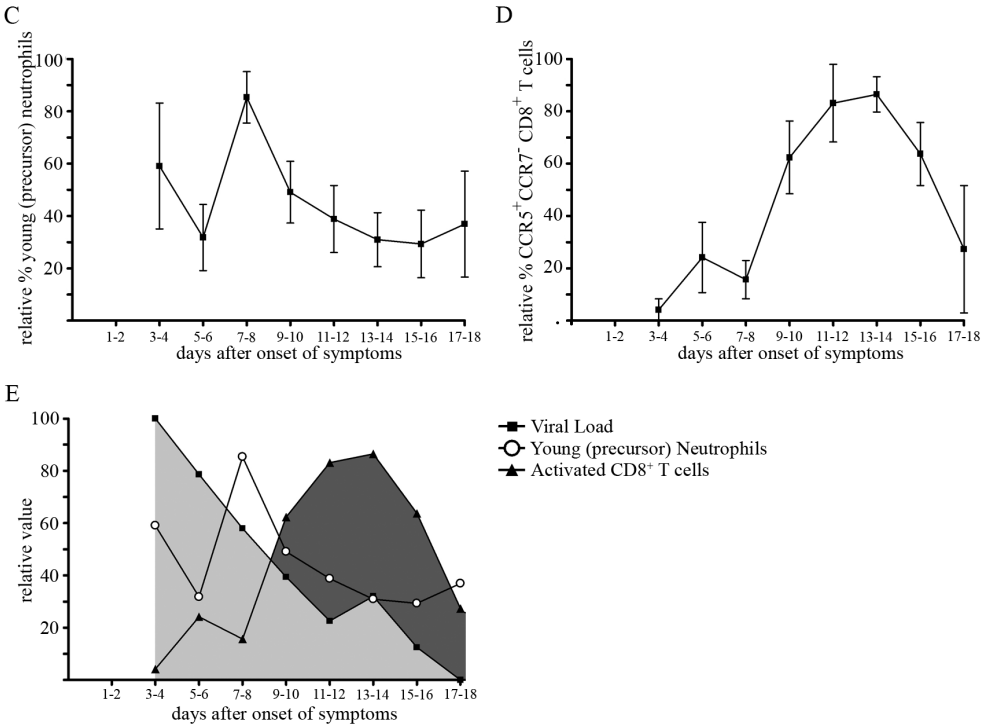
Combined kinetics of viral and immune parameters during RSV infection

The kinetics of the neutrophil precursors and activation of $CD8^+$ T cells combined with the quantitative data on viral load and disease severity were normalized and grouped for every two days to eliminate variations in height of responses and time point of sampling between the patients. The period between first signs of infection, disease manifestation and subsequent admission to the ICU is variable between patients due to thus far unknown parameters (range 2-14 days, average 5.4 days). Viral load was shown to be maximal at admission to the ICU, and disease severity peaked around day 2-3 after admission to the ICU (Figure 6A and B). While the disease severity and viral load were declining, there was an influx of neutrophil precursors in the blood followed by the activation of $CD8^+$ T cells (Figure 6C and D). Upon discharge from the ICU, RSV RNA is still detectable in the nose, the percentage of neutrophil precursors drops to levels seen in healthy controls and also the level of $CD8^+$ T cell activation declined but, remained higher compared to healthy controls. Together these data showed a sequence of events whereby i) viral load peaked before or upon hospital admission, when disease was most severe, ii) neutrophil precursors peaked in blood around day 7-9 after the onset of symptoms while iii) $CD8^+$ T cell responses peaked in peripheral blood towards the end of the ICU stay around day 11-15 after the onset of symptoms.

We did not observe a correlation between the immune response activation (neutrophil precursors and effector T cells), viral load, and the ventilation index. However, we observed a weak correlation between the height of the T cell peak and length of stay. This observation should be confirmed in a larger group of patients in the future.

Figure 6 Dynamics of disease parameters during primary RSV infection in infants





(A) Kinetics of normalized and grouped data of disease severity and (B) viral load in TA during the stay at the ICU. (C) Normalized values of precursor neutrophil percentages and (D) activated $CCR5^{+}CCR7^{-}CD8^{+}$ T cell recruitment into peripheral blood. The error bars represent the standard error of the mean (SEM). (E) Combined dynamics of viral load, neutrophil precursor recruitment and $CD8^{+}$ T cell activation.

Discussion

We explored the dynamics of human neutrophil and $CD8^{+}$ T cell responses during acute severe RSV induced bronchiolitis in infants younger than 6 months of age. To our knowledge this is the first longitudinal analysis correlating parameters of innate and adaptive immune responses with viral load and parameters of disease severity, during a natural primary viral infection in this age group. After admission to the ICU, both viral load (as determined by real-time PCR) and, 2-3 days later, disease severity declined, indicative for the fact that these children were admitted to the ICU with peak values of viral load and disease severity. The decline in viral load in children hospitalized with RSV disease has been described before by other groups (51;52). However, these

studies used quantitative viral culture, or evaluated viral load for a short period after admission (<4 days). We found infectious RSV by viral culture in only 65% of the patients, whereas all patients were RSV positive by real-time PCR. With real-time PCR we could still demonstrate RSV RNA in the nasal area in nearly all patients at discharge, although at lower quantities than at admission (Figure 2B and C). The observed presence of non-infectious virus might indicate that virus is neutralized by antibodies. Interestingly, although serum antibodies are low in peripheral blood after RSV infection in the youngest infants (<6 months), antibody responses in nasal secretions are fast and are similar in magnitude and quality in infants and older children (53). It has recently been shown that during RSV lower respiratory tract infection (LRTI) B lymphocytes and plasma cells are recruited to the lungs of infants at the peak of RSV illness (54). This mucosal B cell activation occurs most likely in a T cell independent way because $CD4^+$ T cells were not found in the lung tissue at the time B cells were abundantly present (54), consistent with our observation that $CD4^+$ T cell responses are hardly detectable in our patients and appear late, i.e. at the time of recovery (data not shown). Interestingly, RSV RNA load declined more rapidly in the lower airways compared to the nasal areas. Because serum IgG antibodies are somewhat more efficient in accessing the lower respiratory tract it is possible that more efficient clearance in the lower airways might indicate a role for IgG (55).

Primary RSV infection is characterized by a strong influx of neutrophils into the airways (13-15). Furthermore, increased numbers of neutrophil precursors in peripheral blood are a characteristic feature during severe inflammatory conditions (40;56). Therefore, the recruitment of bone marrow derived neutrophil progenitors indicated a dramatic involvement of neutrophils during RSV disease and the RSV infection presumably exhausted the banded neutrophil pool in peripheral blood (Figure 4B). Mechanical ventilation can induce a local and systemic inflammatory response and enhanced neutrophil activation and recruitment to the lungs (57). However, also in non-ventilated children with severe RSV disease a strong neutrophil response was observed, demonstrating that marked involvement of neutrophils is a characteristic feature during RSV infection (17). Nevertheless, it is possible that during the first days of respiratory support ventilation induced effects might enhance neutrophil responses and even delay recovery.

T cell activation in peripheral blood was quantified based on a set of antibodies directed against well known activation-associated surface markers and cytotoxic proteins; CD27, CD45RA, CD45RO, CD127, CCR5, CCR7, Perforin, Ki-67 and Granzyme B (GzmB). Based on these parameters we showed that despite the young age of the patients, a robust $CD8^+$ T cell response developed during primary RSV infection. In all patients $CD8^+$ T cell responses peaked between 11-15 days after onset of first symptoms. The phenotype of the $CD8^+$ T cells showed that they were actively dividing cells (Ki67 expression) (48). They acquired the ability to migrate to a peripheral site of inflammation because we observed the reciprocal down regulation of CCR7,

a lymph node homing receptor, and upregulation of CCR5 a chemokine receptor involved in migration of T cells to inflamed tissues. Moreover, the presence of components of the lytic machinery, GzmB and perforin, showed their functional potential (50).

Recently, Miller *et al* described the longitudinal analysis of $CD8^+$ effector and memory T cell responses to live smallpox (Dryvax) and yellow fever virus (YFV-17D) vaccines in adult volunteers (58). These vaccines are attenuated live virus vaccines that cause acute infections. Based on phenotypic analysis ($CD38^+$/HLA-DR^+/Bcl^-2low and Ki-67^+) and HLA-tetramer staining, peak $CD8^+$ T cell effector responses were found at day 15 after vaccination that declined sharply by day 30. YFV was detected in serum by PCR and viral load peaked on day 7 after vaccination and became undetectable by day 11. Peak $CD8^+$ T cell responses based on phenotypic analysis ranged from 2-13 % for YFV and 10-40 % for Dryvax. $CD8^+$ T cell responses detected in infants during natural infection with RSV were very similar to the T cell responses described by Miller after Dryvax and YFV vaccination. First we found the peak of the $CD8^+$ T cell response between day 11 and 15 after first symptoms that occur 3-5 days later than virus inoculation (59). In addition to the similarities in kinetics, we observed a range of responding Th1 $CD8^+$ T cells; between 1 and 40 % of $CD8^+$ T cells in our patient population comparable to the responses in healthy adults. The striking similarity between the kinetics and height of the response between healthy adults and the predominantly < 3 month old infants during the different viral infections is remarkable. The infant immune system is generally considered to be immature, Th2 biased and thought to be weaker compared to adults (21). However, our findings indicate that despite the young age, infants can develop robust $CD8^+$ T cell responses against a respiratory viral infection. In contrast to complete virus eradication in serum observed after YFV vaccination we still detected RSV by PCR in the airways of children at discharge from the ICU which was around day 17 after the onset of symptoms. Hence, in this location virus eradication seemed less efficient in RSV patients compared to systemic viremia in YFV vaccinated individuals. In earlier work we have found that one third of the effector $CD8^+$ T cells of RSV patients, identified by phenotypic analysis, responded with IFN-γ production upon RSV stimulation (15). Also this observation was similar in adults where one third of the $CD8^+$ T cells responded by production of IFN-γ after antigenic stimulation. It is currently unclear why only one third of the effector $CD8^+$ T cells produce IFN-γ upon stimulation with virus antigen. In the infants in our study, the T cell compartment consists mostly of naïve cells, which makes substantial bystander activation unlikely. Also after live vaccine induced $CD8^+$ T cell responses in adults no evidence of bystander T cell activation was observed (58).

All these similarities suggest that the neonatal response to an acute viral infection is comparable to primary T cell responses induced in adults with respect to kinetics, magnitude and function. The sequence of events in these children, with high viral loads and disease parameters upon admission to the ICU while T cell responses are absent, suggest that these

T cells play a minor role in RSV immune pathology. In contrast, massive neutrophil influx in the airways and subsequent recruitment of neutrophil precursors a few days after peak viral load and disease severity indicated that these cells might be important contributors to RSV disease (13-15). A similar conclusion was made by Welliver *et al*. who described lung tissue analysis in autopsy materials from RSV and influenza virus infected children (17). Lymphocytes were hardly detected in the lungs of the children who died from severe RSV LRTI. However, strong neutrophil influxes were found in the autopsy material of these children (17). Our findings confirmed this study by showing that the T cell response develops after the peak of disease, while neutrophils dominated the early response.

It has been shown that neutralizing antibodies and antivirals have no protective ability when given during established disease (32). This observation suggests that lowering viral load during disease has no beneficial effect. Because T cell responses peak at convalescence and therefore do not appear to contribute to pathology, manipulating innate immune parameters might be the only option for disease treatment, while early administered replicating vaccines might induce protective T cell immunity.

Acknowledgements

This work was supported by grant no. OZF-02-004 from the Wilhelmina research Fund, an 'Alexandre Suerman program' MD/PhD grant from the University Medical Center Utrecht, The Netherlands and by a research grant (2008) from the European Society of Clinical Microbiology and Infectious Disease (ESCMID). We thank F.A. Gruppen for excellent technical support.

Reference List

(1) Kohlmeier JE, Woodland DL. Immunity to respiratory viruses. Annu Rev Immunol 2009;27:61-82.
(2) Kawai T, Akira S. Innate immune recognition of viral infection. Nat Immunol 2006 February;7(2):131-7.
(3) Bowie AG, Unterholzner L. Viral evasion and subversion of pattern-recognition receptor signalling. Nat Rev Immunol 2008 December;8(12):911-22.

(4) Claassen EA, van Bleek GM, Rychnavska ZS, de Groot RJ, Hensen EJ, Tijhaar EJ, van EW, van der Most RG. Identification of a CD4 T cell epitope in the pneumonia virus of mice glycoprotein and characterization of its role in protective immunity. Virology 2007 November 10;368(1):17-25.
(5) Hou S, Doherty PC, Zijlstra M, Jaenisch R, Katz JM. Delayed clearance of Sendai virus in mice lacking class I MHC-restricted CD8+ T cells. J Immunol 1992 August 15;149(4):1319-25.
(6) Graham BS, Bunton LA, Wright PF, Karzon DT. Role of T lymphocyte subsets in the pathogenesis of primary infection and rechallenge with respiratory syncytial virus in mice. J Clin Invest 1991 September;88(3):1026-33.
(7) de Jong MD, Simmons CP, Thanh TT, Hien VM, Smith GJ, Chau TN, Hoang DM, Chau NV, Khanh TH, Dong VC, Qui PT, Cam BV, Ha dQ, Guan Y, Peiris JS, Chinh NT, Hien TT, Farrar J. Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. Nat Med 2006 October;12(10):1203-7.
(8) Kobasa D, Jones SM, Shinya K, Kash JC, Copps J, Ebihara H, Hatta Y, Kim JH, Halfmann P, Hatta M, Feldmann F, Alimonti JB, Fernando L, Li Y, Katze MG, Feldmann H, Kawaoka Y. Aberrant innate immune response in lethal infection of macaques with the 1918 influenza virus. Nature 2007 January 18;445(7125):319-23.
(9) Moyron-Quiroz JE, Rangel-Moreno J, Kusser K, Hartson L, Sprague F, Goodrich S, Woodland DL, Lund FE, Randall TD. Role of inducible bronchus associated lymphoid tissue (iBALT) in respiratory immunity. Nat Med 2004 September;10(9):927-34.
(10) Sun J, Madan R, Karp CL, Braciale TJ. Effector T cells control lung inflammation during acute influenza virus infection by producing IL-10. Nat Med 2009 March;15(3):277-84.
(11) Zampieri CA, Sullivan NJ, Nabel GJ. Immunopathology of highly virulent pathogens: insights from Ebola virus. Nat Immunol 2007 November;8(11):1159-64.
(12) Hall CB, Weinberg GA, Iwane MK, Blumkin AK, Edwards KM, Staat MA, Auinger P, Griffin MR, Poehling KA, Erdman D, Grijalva CG, Zhu Y, Szilagyi P. The burden of respiratory syncytial virus infection in young children. N Engl J Med 2009 February 5;360(6):588-98.
(13) Everard ML, Swarbrick A, Wrightham M, McIntyre J, Dunkley C, James PD, Sewell HF, Milner AD. Analysis of cells obtained by bronchial lavage of infants with respiratory syncytial virus infection. Arch Dis Child 1994 November;71(5):428-32.
(14) Smith PK, Wang SZ, Dowling KD, Forsyth KD. Leucocyte populations in respiratory syncytial virus-induced bronchiolitis. J Paediatr Child Health 2001 April;37(2):146-51.
(15) Heidema J, Lukens MV, van Maren WW, van Dijk ME, Otten HG, van Vught AJ, van der Werff DB, van Gestel SJ, Semple MG, Smyth RL, Kimpen JL, van Bleek GM. CD8+ T Cell Responses in Bronchoalveolar Lavage Fluid and Peripheral Blood Mononuclear Cells of Infants with Severe Primary Respiratory Syncytial Virus Infections. J Immunol 2007 December 15;179(12):8410-7.
(16) Johnson JE, Gonzales RA, Olson SJ, Wright PF, Graham BS. The histopathology of fatal untreated human respiratory syncytial virus infection. Mod Pathol 2007 January;20(1):108-19.

(17) Welliver TP, Garofalo RP, Hosakote Y, Hintz KH, Avendano L, Sanchez K, Velozo L, Jafri H, Chavez-Bueno S, Ogra PL, McKinney L, Reed JL, Welliver RC, Sr. Severe human lower respiratory tract illness caused by respiratory syncytial virus and influenza virus is characterized by the absence of pulmonary cytotoxic lymphocyte responses. J Infect Dis 2007 April 15;195(8):1126-36.
(18) Cannon MJ, Openshaw PJ, Askonas BA. Cytotoxic T cells clear virus but augment lung pathology in mice infected with respiratory syncytial virus. J Exp Med 1988 September 1;168(3):1163-8.
(19) DeVincenzo JP, El Saleeby CM, Bush AJ. Respiratory syncytial virus load predicts disease severity in previously healthy infants. J Infect Dis 2005 June 1;191(11):1861-8.
(20) Wright PF, Gruber WC, Peters M, Reed G, Zhu Y, Robinson F, Coleman-Dockery S, Graham BS. Illness severity, viral shedding, and antibody responses in infants hospitalized with bronchiolitis caused by respiratory syncytial virus. J Infect Dis 2002 April 15;185(8):1011-8.
(21) Adkins B, Leclerc C, Marshall-Clarke S. Neonatal adaptive immunity comes of age. Nat Rev Immunol 2004 July;4(7):553-64.
(22) Barrios C, Brawand P, Berney M, Brandt C, Lambert PH, Siegrist CA. Neonatal and early life immune responses to various forms of vaccine antigens qualitatively differ from adult responses: predominance of a Th2-biased pattern which persists after adult boosting. Eur J Immunol 1996 July;26(7):1489-96.
(23) Winkler S, Willheim M, Baier K, Schmid D, Aichelburg A, Graninger W, Kremsner PG. Frequency of cytokine-producing T cells in patients of different age groups with Plasmodium falciparum malaria. J Infect Dis 1999 January;179(1):209-16.
(24) Roman M, Calhoun WJ, Hinton KL, Avendano LF, Simon V, Escobar AM, Gaggero A, Diaz PV. Respiratory syncytial virus infection in infants is associated with predominant Th-2-like response. Am J Respir Crit Care Med 1997 July;156(1):190-5.
(25) Sung RY, Hui SH, Wong CK, Lam CW, Yin J. A comparison of cytokine responses in respiratory syncytial virus and influenza A infections in infants. Eur J Pediatr 2001 February;160(2):117-22.
(26) Legg JP, Hussain IR, Warner JA, Johnston SL, Warner JO. Type 1 and type 2 cytokine imbalance in acute respiratory syncytial virus bronchiolitis. Am J Respir Crit Care Med 2003 September 15;168(6):633-9.
(27) Brandenburg AH, Kleinjan A, van Het LB, Moll HA, Timmerman HH, de Swart RL, Neijens HJ, Fokkens W, Osterhaus AD. Type 1-like immune response is found in children with respiratory syncytial virus infection regardless of clinical severity. J Med Virol 2000 October;62(2):267-77.
(28) de Waal L., Koopman LP, van B, I, Brandenburg AH, Mulder PG, de Swart RL, Fokkens WJ, Neijens HJ, Osterhaus AD. Moderate local and systemic respiratory syncytial virus-specific T-cell responses upon mild or subclinical RSV infection. J Med Virol 2003 June;70(2):309-18.

(29) Bont L, Heijnen CJ, Kavelaars A, Van Aalderen WM, Brus F, Draaisma JT, Geelen SM, van Vught HJ, Kimpen JL. Peripheral blood cytokine responses and disease severity in respiratory syncytial virus bronchiolitis. Eur Respir J 1999 July;14(1):144-9.
(30) The IMpact-RSV Study Group. Palivizumab, a humanized respiratory syncytial virus monoclonal antibody, reduces hospitalization from respiratory syncytial virus infection in high-risk infants. Pediatrics 1998 September;102(3 Pt 1):531-7.
(31) Hall CB, Walsh EE, Hruska JF, Betts RF, Hall WJ. Ribavirin treatment of experimental respiratory syncytial viral infection. A controlled double-blind study in young adults. JAMA 1983 May 20;249(19):2666-70.
(32) Ventre K, Randolph AG. Ribavirin for respiratory syncytial virus infection of the lower respiratory tract in infants and young children.
Cochrane Database Syst Rev 2007;(1):CD000181.
(33) Blom D, Ermers M, Bont L, Van Aalderen WM, van Woensel JB. Inhaled corticosteroids during acute bronchiolitis in the prevention of post-bronchiolitic wheezing.
Cochrane Database Syst Rev 2007;(1):CD004881.
(34) Bont L, Kavelaars A, Heijnen CJ, van Vught AJ, Kimpen JL. Monocyte interleukin-12 production is inversely related to duration of respiratory failure in respiratory syncytial virus bronchiolitis. J Infect Dis 2000 May;181(5):1772-5.
(35) van de Pol AC, Wolfs TF, Jansen NJ, van Loon AM, Rossen JW. Diagnostic value of real-time polymerase chain reaction to detect viruses in young children admitted to the paediatric intensive care unit with lower respiratory tract infection. Crit Care 2006;10(2):R61.
(36) van de Pol AC, van Loon AM, Wolfs TF, Jansen NJ, Nijhuis M, Breteler EK, Schuurman R, Rossen JW. Increased detection of respiratory syncytial virus, influenza viruses, parainfluenza viruses, and adenoviruses with real-time PCR in samples from patients with respiratory symptoms. J Clin Microbiol 2007 July;45(7):2260-2.
(37) van Doornum GJ, Guldemeester J, Osterhaus AD, Niesters HG. Diagnosing herpesvirus infections by real-time amplification and rapid culture. J Clin Microbiol 2003 February;41(2):576-80.
(38) Hierholzer JC, Killington RA. Virus isolation and quantitation. In: Mahy BW, Kagro HO, editors. Virology methods manual.United Kingdom: Academic press limited; 1996. p. 25-46.
(39) Kerst JM, de HM, van der Schoot CE, Slaper-Cortenbach IC, Kleijer M, von dem Borne AE, van Oers RH. Recombinant granulocyte colony-stimulating factor administration to healthy volunteers: induction of immunophenotypically and functionally altered neutrophils via an effect on myeloid progenitor cells. Blood 1993 December 1;82(11):3265-72.
(40) Hubl W, Andert S, Thum G, Ortner S, Bayer PM. Value of neutrophil CD16 expression for detection of left shift and acute-phase response. Am J Clin Pathol 1997 February;107(2):187-96.

(41) Wagner C, Deppisch R, Denefleh B, Hug F, Andrassy K, Hansch GM. Expression patterns of the lipopolysaccharide receptor CD14, and the FCgamma receptors CD16 and CD64 on polymorphonuclear neutrophils: data from patients with severe bacterial infections and lipopolysaccharide-exposed cells. Shock 2003 January;19(1):5-12.
(42) Elghetany MT. Surface antigen changes during normal neutrophilic development: a critical review. Blood Cells Mol Dis 2002 March;28(2):260-74.
(43) Elghetany MT, Ge Y, Patel J, Martinez J, Uhrova H. Flow cytometric study of neutrophilic granulopoiesis in normal bone marrow using an expanded panel of antibodies: correlation with morphologic assessments. J Clin Lab Anal 2004;18(1):36-41.
(44) Forster R, valos-Misslitz AC, Rot A. CCR7 and its ligands: balancing immunity and tolerance. Nat Rev Immunol 2008 May;8(5):362-71.
(45) Galkina E, Thatte J, Dabak V, Williams MB, Ley K, Braciale TJ. Preferential migration of effector CD8+ T cells into the interstitium of the normal lung. J Clin Invest 2005 December;115(12):3473-83.
(46) Sheeran P, Jafri H, Carubelli C, Saavedra J, Johnson C, Krisher K, Sanchez PJ, Ramilo O. Elevated cytokine concentrations in the nasopharyngeal and tracheal secretions of children with respiratory syncytial virus disease. Pediatr Infect Dis J 1999 February;18(2):115-22.
(47) Kaech SM, Tan JT, Wherry EJ, Konieczny BT, Surh CD, Ahmed R. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. Nat Immunol 2003 December;4(12):1191-8.
(48) Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U, Stein H. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J Immunol 1984 October;133(4):1710-5.
(49) Kagi D, Ledermann B, Burki K, Seiler P, Odermatt B, Olsen KJ, Podack ER, Zinkernagel RM, Hengartner H. Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice. Nature 1994 May 5;369(6475):31-7.
(50) Peters PJ, Borst J, Oorschot V, Fukuda M, Krahenbuhl O, Tschopp J, Slot JW, Geuze HJ. Cytotoxic T lymphocyte granules are secretory lysosomes, containing both perforin and granzymes. J Exp Med 1991 May 1;173(5):1099-109.
(51) Hall CB, Douglas RG, Jr., Geiman JM. Quantitative shedding patterns of respiratory syncytial virus in infants. J Infect Dis 1975 August;132(2):151-6.
(52) van Woensel JB, Lutter R, Biezeveld MH, Dekker T, Nijhuis M, Van Aalderen WM, Kuijpers TW. Effect of dexamethasone on tracheal viral load and interleukin-8 tracheal concentration in children with respiratory syncytial virus infection. Pediatr Infect Dis J 2003 August;22(8):721-6.
(53) Jensen IP, Thisted E, Glikmann G, Obel N, Kofoed PE, Sambo M, Valerius NH, Mordhorst CH. Secretory IgM and IgA antibodies to respiratory syncytial virus in nasopharyngeal aspirates: a diagnostic supplement to antigen detection. Clin Diagn Virol 1997 November;8(3):219-26.

(54) Reed JL, Welliver TP, Sims GP, McKinney L, Velozo L, Avendano L, Hintz K, Luma J, Coyle AJ, Welliver RC, Sr. Innate immune signals modulate antiviral and polyreactive antibody responses during severe respiratory syncytial virus infection. J Infect Dis 2009 April 15;199(8):1128-38.
(55) Siber GR, Leombruno D, Leszczynski J, McIver J, Bodkin D, Gonin R, Thompson CM, Walsh EE, Piedra PA, Hemming VG, . Comparison of antibody concentrations and protective activity of respiratory syncytial virus immune globulin and conventional immune globulin. J Infect Dis 1994 June;169(6):1368-73.
(56) Orr Y, Taylor JM, Bannon PG, Geczy C, Kritharides L. Circulating CD10-/CD16low neutrophils provide a quantitative index of active bone marrow neutrophil release. Br J Haematol 2005 November;131(4):508-19.
(57) Frank JA, Matthay MA. Science review: mechanisms of ventilator-induced injury. Crit Care 2003 June;7(3):233-41.
(58) Miller JD, van der Most RG, Akondy RS, Glidewell JT, Albott S, Masopust D, Murali-Krishna K, Mahar PL, Edupuganti S, Lalor S, Germon S, Del RC, Mulligan MJ, Staprans SI, Altman JD, Feinberg MB, Ahmed R. Human effector and memory CD8+ T cell responses to smallpox and yellow fever vaccines. Immunity 2008 May;28(5):710-22.
(59) Johnson KM, Chanock RM, Rifkind D, Kravetz HM, Knight V. Respiratory syncytial virus. IV. Correlation of virus shedding, serologic response, and illness in adult volunteers. JAMA 1961 May 27;176:663-7.

CHAPTER 10

General discussion

Discussion

Epidemiology of respiratory viruses

The introduction of molecular methods (e.g. real-time PCR) for the detection of respiratory viruses in the work-up of immunocompetent hospitalized patients with lower respiratory tract infection (LRTI) has greatly increased our insight in respiratory virus epidemiology. Before the availability of PCR, our understanding of the epidemiology of respiratory viruses was based on viral detection methods with less sensitivity, such as the conventional methods viral culture and direct immunofluorescence (DIF). With viral culture and DIF only four respiratory viruses can routinely be detected: respiratory syncytial virus (RSV), influenza virus (IV), parainfluenza virus (PIV) and adenovirus (AdV). As a consequence, these four viruses were previously considered the main players in viral LRTI in young children (1;2). With the availability of molecular methods this concept has changed considerably. Human rhinovirus (hRV), human coronavirus (hCoV), human metapneumovirus (hMPV), and human bocavirus (hBoV), have all been found frequently in patients with LRTI (3-7). The newly discovered KI polyomavirus (KIPyV) and WU polyomavirus (WUPyV), have been found at lower frequencies, and their pathogenic role is still a matter of debate (7).

Although it appears beneficial that molecular methods are available to test with high sensitivity for a broader range of viruses, there is no consensus among laboratories and clinicians which diagnostic methods for respiratory viruses should be used routinely in the hospital setting. However, over the last few years, many laboratories switched from conventional methods (i.e. viral culture and DIF) to molecular methods (i.e. real-time PCR) (8-10). At the University Medical Center (UMC) Utrecht real-time PCR was implemented during the winter of 2004-2005, and it was used in addition to conventional tests in the routine work-up of inpatients with significant respiratory symptoms. During this winter a great increase was observed in the number of viruses detected. Overall, the diagnostic yield of RSV, IV, PIV, and AdV was increased from roughly 25% to 45% in children, and from 5% to 35% in adults [Chapter 2], indicating that real-time PCR was more sensitive than conventional methods for these viruses. In pediatric patients with severe LRTI at the pediatric intensive care unit (PICU), we used molecular methods to detect RSV, IV, PIV, AdV, hRV, hCoV, and hMPV and found an even higher increase in the number of viruses detected. In the latter population the frequency of RSV, IV, PIV, and AdV detection with conventional tests was high with 50%, however real-time PCR increased the diagnostic yield to nearly 100% [Chapter 3 and Chapter 6].

The increased number of viruses detected at the PICU has two implications. First, it changed our view on the epidemiology of respiratory viruses. HRV, for example, used to be detected

infrequently with conventional methods, simply because hRV does not grow well in viral culture, and hRV DIF tests are not commercially available. With real-time PCR, however hRV was found to be the second most common respiratory virus in children under 5 years of age at our PICU [Chapter 3, Chapter 9]. This finding is consistent with other recent studies, that detected hRV as the first or second most common cause in patients with LRTI (11-13).
Furthermore, multiple viruses in one single patient were commonly identified. In total, 35% of PICU patients with LRTI were found to be infected with multiple viruses with real-time PCR, compared to 0% with conventional methods. This finding is in agreement with previous studies which, depending on setting, season, and number of viruses tested, identified multiple viral infections in 20-30% of LRTI patients (5;6;14;15). The increase in the number of viral (co-) infections is remarkable. However, whether all detected viruses play a role in the etiology of disease remains to be determined.

Second, the close to 100% positivity rate in young children with LRTI at the PICU, changes the interpretation of a positive viral test. Before the introduction of real-time PCR only a limited number of patients was found to be virus infected, and in these patients the virus was often regarded a sufficient explanation of symptoms. Nowadays, with all PICU patients with LRTI being virus-positive by real-time PCR, the question arises whether all viral detections should be regarded as true etiological agents.

Etiologic role of respiratory viruses

In the past, any respiratory virus detected in the airways of patients with LRTI was considered to be a causal agent, however with the introduction of molecular methods this concept has changed (16;17). Since real-time PCR is more sensitive it can detect smaller quantities of virus, and after viral infection, virus shedding is now detected for longer periods of time. Viral (co-)infections are found so often with PCR that the question arises whether viruses can also be present as coincidental bystanders. The problem of detecting bystander virus is potentially greater with PCR than with conventional test because PCR does not necessarily detect intact and/or infectious virus. Any viral DNA/RNA fragment against which the PCR is targeted will generate a positive result.

The pathological role of respiratory viruses can be investigated by relating the presence of a virus to the presence of disease (i.e. case-control studies). We used this type of study to find an association between the prevalence of the newly discovered viruses hBoV, KIPyV, and WUPyV, and the presence of LRTI. HBoV, KIPyV, and WUPyV were found in 0-5% of PICU patients with LRTI, but also in 0-5% of PICU controls without LRTI [Chapter 5]. In nearly all positive LRTI cases, a second virus was found as a co-infection. These observations argue against a pathological role of the newly detected viruses in severe LRTI.

Furthermore, the pathogenic role of viruses in individual patients can be examined by assessing viral RNA/DNA quantities. If high viral load is associated with severe symptoms, and low load with mild or no symptoms, this could be a way to differentiate acute infection from asymptomatic carriership. In our study on hBoV, KIPyV, and WUPyV the viral load of the three viruses was higher in LRTI patients than in controls. Although this finding was not significant due to the low number of positive patients, it could be an indication that hBoV, KIPyV, and WUPyV found in high loads are pathogenic, and minute amounts of these viruses in controls indicate carriership without LRTI symptoms. In the future, additional viral load studies on the clinical significance of the different respiratory viruses (i.e. hRV, multiple viral infections, new viruses such as hBoV, KIPyV, and WUPyV) are needed to elucidate their role in LRTI patients. It will be important to include appropriate reference groups, i.e. patients with mild LRTI at the general ward, patients with RTI at home, and asymptomatic controls, and all groups need to be matched for age, season, and sampling procedure.

Finally, the course of viral load in time might provide important information on the pathogenic role of a virus. If a virus is initially found in high quantities, and this quantity diminishes upon resolution of symptoms, an etiologic role for this virus becomes more likely. We evaluated the course of viral load in patients with RSV LRTI at the PICU. We started by showing the validity of quantitative respiratory PCR detection in mucus samples. We focused on RSV as a model virus, since historically RSV is regarded as the predominant cause of pediatric LRTI. We showed that quantitative real-time PCR for RSV is reliable for the identification of viral load differences in the order of a magnitude of 5 cycle threshold (Ct) values or more [Chapter 8]. Differences of 5 Ct values correspond to approximately 30-fold changes in viral load; this amount of change will likely be of interest clinically, since upon viral infection viral load increases approximately 100-fold in a matter of hours (18). We found that, if patients are sampled early in the course of infection, viral load in the nose gives a reliable estimate of viral load in the lower airways.

Quantitative real-time PCR was used to relate the RSV RNA load to disease severity and activation of the innate and adaptive immune system of PICU patients. We did not find a relation between magnitudes of viral load, or activity of the immune system, and disease severity as measured in ventilation parameters [chapter 9]. However, when we looked at viral and immune kinetics, we found that viral load was high upon admission and declined parallel to resolution of symptoms. The order of events was as follows: first viral load peaked (in nose and trachea), followed by neutrophil influx from the bone marrow (into the blood), and finally followed by late CD8+ T-cel activation (in the blood). From the sequence of events we conclude that CD8+ T cells responses initiated during primary RSV infections are unlikely to contribute to disease when it is most severe. A causal role for RSV is more likely, since viral load was high upon admission an decreased parallel to resolution of symptoms. However, since the magnitude of viral load was not related to severity of disease, we cannot explain why some patients at the PICU had to stay longer than others. To our knowledge, this was the

first time that viral RNA load and immune response kinetics were followed over time in infants with natural viral infection, and future studies are required to confirm our observations.

Clinical implications of viral testing

Over the past few years it has become clear that the development of molecular methods, such as real-time PCR, has greatly increased our insight in the epidemiology of respiratory viruses. Quantitative real-time PCR provides a valuable tool to study the pathogenicity of viruses, and thanks to the availability of molecular methods new viruses are continuously being discovered. However, what this means for clinicians treating children with LRTI at the PICU remains unclear. On clinical ground, viral and bacterial LRTI are difficult to differentiate. Rapid testing with DIF has been used at the PICU for decades to identify viruses in LRTI patients. Viruses identified may be considered a sufficient explanation for respiratory symptoms, and antibiotics might not be prescribed (19-22). This practice is supported by epidemiological studies showing that RSV positive patients generally have a low risk of a significant bacterial co-infection, and prescription of antibiotics in patients with a clinical suspicion of bronchiolitis is ineffective (23-26). However, the actual influence of viral testing in the clinical setting on antibiotics treatment is controversial, with several studies on this issue showing contradictory results (19;27-31). Even though evidence is lacking, DIF tests are still being used routinely in the work-up of LRTI patients at the PICU, and additional molecular methods for testing broad ranges of respiratory viruses are increasingly implemented (8-10;32;33).

The implementation of real-time PCR is accompanied by a huge increase in costs. Real-time PCR is much more expensive than DIF assays (8;9;34;35). As described above the UMC Utrecht implemented real-time PCR during the winter of 2004-2005, as a routine diagnostic test additional to conventional tests (viral culture and DIF). The next year, 2006, a standard "respiratory test panel" was requested 60 times at the PICU, and each panel now included real-time PCR for a broad range of respiratory viruses (12 different viruses), for 650 euros per panel. As a consequence, an additional amount of 40.000 euros was spent on viral testing, for the PICU only. The total increase in costs for all patients with LRTI at our center was more than 4 times as large. This huge increase in costs can only be justified if the performance of real-time PCR actually impacts the management of PICU patients.

To investigate the impact of real-time PCR on antibiotic use in PICU patients with LRTI we designed questionnaires with theoretical descriptions of ventilated LRTI patients. Nearly 100 clinicians in the Netherlands and Germany were questioned, and we found that the percentage of clinicians that would prescribe antibiotics for the paper cases decreased when positive real-time PCR results were made available [chapter 6]. This prompted us to continue

with a prospective study, questioning clinicians at our PICU on antibiotic prescription of LRTI patients during two winter seasons. We wanted to know whether the introduction of real-time PCR, in addition to DIF testing, changed antibiotic use. The prescription of antibiotics was assessed before, and one hour after revealing the real-time PCR result. Real-time PCR, additional to DIF, did not change antibiotic use at all in PICU patients with LRTI [chapter 6]. Apparently, in real-life the situation is different than in a hypothetical case scenario, demonstrating that clinicians seem reluctant to stop antibiotic prescriptions in the severely ill LRTI patients .

In retrospect, the lack of impact of real-time PCR at the PICU is understandable, since by using this method for a broad range of viruses virtually all LRTI patients are found to harbor at least one respiratory virus. This makes it no longer a discriminative test. When clinicians were questioned as to why they did not change antibiotic use in a patient that was virus positive with real-time PCR, the majority reported that they had a high clinical suspicion of a bacterial infections and they waited for the bacterial cultures to come back negative. Given the results of our studies, we believe that at this moment there is no evidence that real-time PCR needs to be included as a standard tool in the work-up of LRTI patients at the PICU, other than for research purposes.

In the future, this situation at the PICU might change for a number of reasons. First, the etiologic role of (some of) the respiratory viruses detected by real-time PCR might become more clear. For example, certain viruses (i.e. hRV, hCoV, hBoV) may be found to be only associated with mild respiratory disease, and these can then be left out of the test panels for PICU patients. On the other hand, research is going on to determine whether certain combinations of viral co-infections can be recognized as sufficient explanations for severe LRTI, and in patients with these viruses antibiotics might not be prescribed (14;36-38).

Second, quantitative PCR might become an important tool to detect viral load. In this thesis we show that quantitative PCR in respiratory samples is feasible, however we could not find a relation between viral load and disease severity in patients with RSV LRTI. We did, however, find preliminary evidence that viral load is important in the newly discovered viruses hBoV, KIPyV, and WUPyV. Future studies should focus further on the clinical relevance of viral load detection in patients with LRTI and controls. Possibly, PCR is too sensitive and therefore detects clinically irrelevant viral carriership. This problem could be solved by determining a cut-off value, e.g. a maximum number of cycles above which samples are considered positive.

Third, viruses are not the only pathogens that merit detection by a quantitative and sensitive method such as real-time PCR. Many clinicians at the PICU reported that they did not want to change their antibiotic treatment based on the detection of viruses, as long as a bacterial infection was not ruled out. Real-time PCR for bacteria is almost never used, and this seems a

missed opportunity. Reliable real-time PCR assays for bacterial pathogens need to be developed to make real-time PCR an even more comprehensive tool to investigate the etiology of LRTI at the PICU. Asymptomatic bacterial colonization can be expected to make the interpretation of positive bacterial test difficult. However, this problem can be overcome if a correlation between the bacteria load and pathogenicity can be established.

Fourth, the sensitivity of real-time PCR will be beneficial when effective anti-viral treatment becomes available in the future. When anti-viral treatment is possible, the fact that we have implemented and validated real-time PCR for respiratory viruses at our hospital will be a great advantage. Ideally, PCR logistics should be even further improved. A turn-around time of less than 24 hours is expected to improve the clinical impact of real-time PCR results.

Finally, in future viral outbreaks the sensitivity and speed of real-time PCR may become of great value. We assessed the impact of real-time PCR as a tool to identify RSV transmission among PICU patients. In our surveillance study we found no transmission of RSV with real-time PCR. Current practice seemed adequate to limit RSV spread [chapter 7] at our PICU with strict infection control measures. However, this does not have to be the case with all respiratory viruses, or in all clinical settings (i.e. it might be different in the emergency department or general wards). Therefore, further research on viral transmission remains necessary (39;40).

The clinical impact of real-time PCR on anti-viral therapy and infection control has been clearly shown in the current pandemic of the new H1N1 influenza A virus (41). Real-time PCR assays were developed promptly, which the Center of Disease Control distributed around the world to make global surveillance possible (42-44). Thanks to rapid PCR based surveillance in numerous settings (i.e. family practice, hospital wards, PICUs), viral inhibitors can be prescribed in a timely fashion, and rational infection control measures can be taken. With this example in mind, we should be careful not to discard real-time PCR because of its limited clinical implication at the PICU. We need to continue to develop and validate real-time PCR assays to improve the care of patients with severe LRTI.

Conclusions

Molecular methods such as real-time PCR were shown to be sensitive and reliable to investigate the epidemiology of respiratory viruses in patients with LRTI at the PICU. Using real-time PCR, respiratory viruses were found in nearly 100% of PICU patients with LRTI, a two-fold increase in detection compared to the detection rate using conventional viral tests (viral culture and

DIF). However, the implementation of real-time PCR, additional to DIF, as a standard viral test at the PICU did not impact antibiotic use in LRTI patients. As a consequence, there is currently no need to perform real-time PCR routinely at the PICU. This could change for a number of reasons. In the future reliable bacterial PCR assays may become available to exclude bacterial superinfection. Furthermore, real-time PCR may prove valuable to allocate and monitor antiviral therapy and infection control in outbreaks of new pathogenic viruses. And finally, from a scientific point of view, it is important to continue to implement and develop quantitative real-time PCR assays to further elucidate the etiologic role of respiratory viruses.

Reference List

(1) Henrickson KJ, Hoover S, Kehl KS, Hua W. National disease burden of respiratory viruses detected in children by polymerase chain reaction. Pediatr Infect Dis J 2004 January;23 (1 Suppl):S11-S18.

(2) Iwane MK, Edwards KM, Szilagyi PG, Walker FJ, Griffin MR, Weinberg GA, Coulen C, Poehling KA, Shone LP, Balter S, Hall CB, Erdman DD, Wooten K, Schwartz B. Population-based surveillance for hospitalizations associated with respiratory syncytial virus, influenza virus, and parainfluenza viruses among young children. Pediatrics 2004 June;113(6):1758-64.

(3) Allander T, Jartti T, Gupta S, Niesters HG, Lehtinen P, Osterback R, Vuorinen T, Waris M, Bjerkner A, Tiveljung-Lindell A, van den Hoogen BG, Hyypia T, Ruuskanen O. Human bocavirus and acute wheezing in children. Clin Infect Dis 2007 April 1;44(7):904-10.

(4) Arnold JC, Singh KK, Spector SA, Sawyer MH. Undiagnosed respiratory viruses in children. Pediatrics 2008 March;121(3):e631-e637.

(5) Jartti T, Lehtinen P, Vuorinen T, Ruuskanen O. Bronchiolitis: age and previous wheezing episodes are linked to viral etiology and atopic characteristics. Pediatr Infect Dis J 2009 April;28(4):311-7.

(6) Jennings LC, Anderson TP, Werno AM, Beynon KA, Murdoch DR. Viral etiology of acute respiratory tract infections in children presenting to hospital: role of polymerase chain reaction and demonstration of multiple infections. Pediatr Infect Dis J 2004 November;23(11):1003-7.

(7) Sloots TP, Whiley DM, Lambert SB, Nissen MD. Emerging respiratory agents: new viruses for old diseases? J Clin Virol 2008 July;42(3):233-43.

(8) Henrickson KJ. Advances in the laboratory diagnosis of viral respiratory disease. Pediatr Infect Dis J 2004 January;23(1 Suppl):S6-10.

(9) Leland DS, Ginocchio CC. Role of cell culture for virus detection in the age of technology. Clin Microbiol Rev 2007 January;20(1):49-78.

(10) Mackay IM. Real-time PCR in the microbiology laboratory. Clin Microbiol Infect 2004 March;10(3):190-212.
(11) Jartti T, Lehtinen P, Vuorinen T, Osterback R, van den HB, Osterhaus AD, Ruuskanen O. Respiratory picornaviruses and respiratory syncytial virus as causative agents of acute expiratory wheezing in children. Emerg Infect Dis 2004 June;10(6):1095-101.
(12) Louie JK, Roy-Burman A, Guardia-Labar L, Boston EJ, Kiang D, Padilla T, Yagi S, Messenger S, Petru AM, Glaser CA, Schnurr DP. Rhinovirus associated with severe lower respiratory tract infections in children. Pediatr Infect Dis J 2009 April;28(4):337-9.
(13) Miller EK, Lu X, Erdman DD, Poehling KA, Zhu Y, Griffin MR, Hartert TV, Anderson LJ, Weinberg GA, Hall CB, Iwane MK, Edwards KM. Rhinovirus-associated hospitalizations in young children. J Infect Dis 2007 March 15;195(6):773-81.
(14) Aberle JH, Aberle SW, Pracher E, Hutter HP, Kundi M, Popow-Kraupp T. Single versus dual respiratory virus infections in hospitalized infants: impact on clinical course of disease and interferon-gamma response. Pediatr Infect Dis J 2005 July;24(7):605-10.
(15) Drews AL, Atmar RL, Glezen WP, Baxter BD, Piedra PA, Greenberg SB. Dual respiratory virus infections. Clin Infect Dis 1997 December;25(6):1421-9.
(16) Gardner PS. Virus infections and respiratory disease of childhood. Arch Dis Child 1968 December;43(232):629-45.
(17) Jartti T, Jartti L, Peltola V, Waris M, Ruuskanen O. Identification of respiratory viruses in asymptomatic subjects: asymptomatic respiratory viral infections. Pediatr Infect Dis J 2008 December;27(12):1103-7.
(18) Falsey AR, Formica MA, Treanor JJ, Walsh EE. Comparison of quantitative reverse transcription-PCR to viral culture for assessment of respiratory syncytial virus shedding. J Clin Microbiol 2003 September;41(9):4160-5.
(19) Bonner AB, Monroe KW, Talley LI, Klasner AE, Kimberlin DW. Impact of the rapid diagnosis of influenza on physician decision-making and patient management in the pediatric emergency department: results of a randomized, prospective, controlled trial. Pediatrics 2003 August;112(2):363-7.
(20) Vega R. Rapid viral testing in the evaluation of the febrile infant and child. Curr Opin Pediatr 2005 June;17(3):363-7.
(21) Low D. Reducing antibiotic use in influenza: challenges and rewards. Clin Microbiol Infect 2008 April;14(4):298-306.
(22) Halonen P, Herholzer J, Ziegler T. Advances in the diagnosis of respiratory virus infections. Clin Diagn Virol 1996 May;5(2-3):91-100.
(23) Levine DA, Platt SL, Dayan PS, Macias CG, Zorc JJ, Krief W, Schor J, Bank D, Fefferman N, Shaw KN, Kuppermann N. Risk of serious bacterial infection in young febrile infants with respiratory syncytial virus infections. Pediatrics 2004 June;113(6):1728-34.
(24) Titus MO, Wright SW. Prevalence of serious bacterial infections in febrile infants with respiratory syncytial virus infection. Pediatrics 2003 August;112(2):282-4.

(25) Kneyber MC, van Woensel JB, Uijtendaal E, Uiterwaal CS, Kimpen JL. Azithromycin does not improve disease course in hospitalized infants with respiratory syncytial virus (RSV) lower respiratory tract disease: a randomized equivalence trial. Pediatr Pulmonol 2008 February;43(2):142-9.
(26) Spurling GK, Fonseka K, Doust J, Del MC. Antibiotics for bronchiolitis in children. Cochrane Database Syst Rev 2007;(1):CD005189.
(27) Doan QH, Kissoon N, Dobson S, Whitehouse S, Cochrane D, Schmidt B, Thomas E. A randomized, controlled trial of the impact of early and rapid diagnosis of viral infections in children brought to an emergency department with febrile respiratory tract illnesses. J Pediatr 2009 January;154(1):91-5.
(28) Noyola DE, Demmler GJ. Effect of rapid diagnosis on management of influenza A infections. Pediatr Infect Dis J 2000 April;19(4):303-7.
(29) Poehling KA, Zhu Y, Tang YW, Edwards K. Accuracy and impact of a point-of-care rapid influenza test in young children with respiratory illnesses. Arch Pediatr Adolesc Med 2006 July;160(7):713-8.
(30) Sharma V, Dowd MD, Slaughter AJ, Simon SD. Effect of rapid diagnosis of influenza virus type a on the emergency department management of febrile infants and toddlers. Arch Pediatr Adolesc Med 2002 January;156(1):41-3.
(31) Woo PC, Chiu SS, Seto WH, Peiris M. Cost-effectiveness of rapid diagnosis of viral respiratory tract infections in pediatric patients. J Clin Microbiol 1997 June;35(6):1579-81.
(32) Bordley WC, Viswanathan M, King VJ, Sutton SF, Jackman AM, Sterling L, Lohr KN. Diagnosis and testing in bronchiolitis: a systematic review. Arch Pediatr Adolesc Med 2004 February;158(2):119-26.
(33) Madeley CR, Peiris JS. Methods in virus diagnosis: immunofluorescence revisited. J Clin Virol 2002 August;25(2):121-34.
(34) Ieven M. Currently used nucleic acid amplification tests for the detection of viruses and atypicals in acute respiratory infections. J Clin Virol 2007 December;40(4):259-76.
(35) Oosterheert JJ, van Loon AM, Schuurman R, Hoepelman AI, Hak E, Thijsen S, Nossent G, Schneider MM, Hustinx WM, Bonten MJ. Impact of rapid detection of viral and atypical bacterial pathogens by real-time polymerase chain reaction for patients with lower respiratory tract infection. Clin Infect Dis 2005 November 15;41(10):1438-44.
(36) Semple MG, Cowell A, Dove W, Greensill J, McNamara PS, Halfhide C, Shears P, Smyth RL, Hart CA. Dual infection of infants by human metapneumovirus and human respiratory syncytial virus is strongly associated with severe bronchiolitis. J Infect Dis 2005 February 1;191(3):382-6.
(37) Richard N, Komurian-Pradel F, Javouhey E, Perret M, Rajoharison A, Bagnaud A, Billaud G, Vernet G, Lina B, Floret D, Paranhos-Baccala G. The impact of dual viral infection in infants admitted to a pediatric intensive care unit associated with severe bronchiolitis. Pediatr Infect Dis J 2008 March;27(3):213-7.

(38) van Woensel JB, Bos AP, Lutter R, Rossen JW, Schuurman R. Absence of human metapneumovirus co-infection in cases of severe respiratory syncytial virus infection. Pediatr Pulmonol 2006 September;41(9):872-4.
(39) Gagneur A, Vallet S, Talbot PJ, Legrand-Quillien MC, Picard B, Payan C, Sizun J. Outbreaks of human coronavirus in a pediatric and neonatal intensive care unit. Eur J Pediatr 2008 December;167(12):1427-34.
(40) Hall CB, Lieberthal AS. Viral testing and isolation of patients with bronchiolitis. Pediatrics 2007 October;120(4):893-4.
(41) Fraser C, Donnelly CA, Cauchemez S, Hanage WP, Van K, Hollingsworth TD, Griffin J, Baggaley RF, Jenkins HE, Lyons EJ, Jombart T, Hinsley WR, Grassly NC, Balloux F, Ghani AC, Ferguson NM, Rambaut A, Pybus OG, Lopez-Gatell H, pluche-Aranda CM, Chapela IB, Zavala EP, Guevara DM, Checchi F, Garcia E et al. Pandemic Potential of a Strain of Influenza A (H1N1) : Early Findings. Science 2009 May 14.
(42) Baden LR, Drazen JM, Kritek PA, Curfman GD, Morrissey S, Campion EW. H1N1 influenza A disease--information for health professionals. N Engl J Med 2009 June 18;360(25):2666-7.
(43) Whiley DM, Bialasiewicz S, Bletchly C, Faux CE, Harrower B, Gould AR, Lambert SB, Nimmo GR, Nissen MD, Sloots TP. Detection of novel influenza A(H1N1) virus by real-time RT-PCR. J Clin Virol 2009 June 6.
(44) Dawood FS, Jain S, Finelli L, Shaw MW, Lindstrom S, Garten RJ, Gubareva LV, Xu X, Bridges CB, Uyeki TM. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. N Engl J Med 2009 June 18;360(25):2605-15.

CHAPTER 11

Summery / samenvatting

Summary

Lower respiratory tract infections (LRTIs) in infants and young children are a major public health problem (introduction; **chapter 1**). Syndromes of LRTI include bronchiolitis and pneumonia, and historically bronchiolitis is believed to be associated with a viral infection and pneumonia with a bacterial infection. However, on clinical ground alone, a distinction between viral and bacterial LRTI cannot be made. In infants and children, it is generally believed that the majority of LRTIs are of viral origin. To make an etiological viral diagnosis conventional tests (viral culture and direct immunofluorescence (DIF)) have been used for decades. Recently, molecular methods such as real-time PCR are being implemented in addition to conventional methods, to test more sensitively for a broader range of viruses. This thesis investigates the clinical impact of introducing real-time PCR in the work-up of immunocompetent infants and young children with LRTI at the pediatric intensive care unit (PICU). First, the prevalence of respiratory viruses was investigated with the use of real-time PCR, and compared with the prevalence of respiratory viruses found with conventional methods (viral culture and DIF). Second, the influence of real-time PCR, when used in addition to DIF, on antibiotics use in PICU patients with LRTI was assessed. Third, we determined whether quantitative real-time PCR in respiratory samples can be used to gain insight in the pathogenic role of respiratory syncytial virus (RSV) in LRTI.

Detection of respiratory viruses

In **chapter 2** the sensitivity and specificity of conventional respiratory viral tests (viral culture and DIF) for RSV, influenza virus (IV), parainfluenza virus (PIV), and adenovirus (AdV) was compared with real-time PCR as the reference method, in 267 samples from hospitalized children and adults with respiratory symptoms. Overall, the use of real-time PCR increased the diagnostic yield of these respiratory viruses from 24% to 43% in children, and from 3.5% to 36% in adults. For most viruses, viral load in samples positive by both real-time PCR and conventional tests was higher than viral load in samples that were positive by real-time PCR only, confirming a lower sensitivity of conventional tests compared to real-time PCR. Overall, the sensitivity of conventional tests was around 30-60% in children and 0-20% in adults, when compared to real-time PCR as the reference method. The specificity of conventional method was near 100% (i.e. all conventional test positives were confirmed by real-time PCR). In conclusion, real-time PCR considerably increased the diagnostic yields for RSV, IV, PIV, and AdV, and thereby has a potential befit for the initiation of optimal patient management.

Subsequently, in **chapter 3** the implementation of real-time PCR in addition to conventional methods was investigated in 23 infants and children under five years of age with respiratory

failure due to LRTI at the PICU. With real-time PCR the prevalence RSV, IV, PIV, AdV, human rhinovirus (hRV), human coronavirus (hCoV) 229E, OC43, and NL63, and human metapneumovirus (hMPV) was determined. By screening for a broad range of viruses at the PICU we found an even higher increase in the number of viruses detected. The frequency of viral detection with conventional methods was high with 48%, however real-time PCR increased the diagnostic yield to 96%. More than one virus was detected in 35% of children.
We concluded that real-time PCR for respiratory viruses is a sensitive and reliable method in PICU patients with lower respiratory tract infection, increasing the diagnostic yield twofold compared to conventional methods.

In **chapter 4**, we evaluated the sensitivity and specificity of conventional respiratory viral tests compared to molecular methods in a systematic review of current literature.
We searched Medline, Embase, and bibliographies of relevant articles for studies that compared conventional tests and PCR for the diagnosis of respiratory viruses in hospitalized infants and children with respiratory symptoms. Thirty-three publications (10,297 patients) were analyzed and viral diagnostic approaches were found to vary considerably. We could not reach a definite conclusion on the sensitivity of conventional methods versus PCR, because study results were too heterogeneous. The sensitivity of conventional tests varied between 0.00 and 1.00.
It is therefore important to develop international standards of viral testing, to ensure adequate sensitivity of viral test methods in every institution worldwide.

Finally, in **chapter 5**, real-time PCR was used to determine the prevalence of three viruses that were recently newly discovered: human bocavirus (hBoV), KI polyomavirus (KIPyV), and WU polyomavirus (WUPyV). Patients under five years of age with LRTI at the PICU (n=78) were included, as was a control group under 18 years of age of PICU patients without LRTI (n=83). The prevalence of hBoV, KIPyV, and WUPyV was 5.1%, 0%, and 2.6%, respectively, in children with LRTI and 4.8%, 4.8%, and 2.4%, respectively, in those without LRTI. Nearly all LRTI patients with hBoV, KIPyV, or WUPyV had a second virus co-detected. Notably, one child with LRTI had a hBoV single infection with a high viral load. Interestingly, the viral load of hBoV, KIPyV, and WUPyV was higher in patients than in controls, although not significantly due to the low number of positives. Possibly, these viruses are pathogenic when found in high viral load, and asymptomatic when found in lower load in the control patients. In conclusion, the low prevalences of hBoV, KIPyV, and WUPyV, both in patients and controls, suggested that these agents are unlikely to be a major cause of LRTI at the PICU. Additional studies are needed to determine whether quantitative viral detection can be used to differentiate acute hBoV/KIPyV/WUPyV infection from asymptomatic carriership.

Impact on patient management

In **chapter 6**, the impact of respiratory virus real-time PCR, in additional to DIF, on antibiotic use of LRTI patients at the PICU is evaluated in a paper case study, followed by a prospective study. In the prospective study children under five years of age were tested by DIF and PCR. Positive DIF results were reported at the end of the first working-day. Physicians reported antibiotic treatment on the second working-day. After informing them of the PCR result antibiotic treatment was reevaluated. The paper case study (94 respondents in the Netherlands and Germany) showed that revealing positive real-time PCR results decreased antibiotic use ($p<0.001$). In the prospective real-life study 38 children were included, of which 19 (50%) were positive by DIF. Twelve (63%) of the 19 DIF-negative patients were treated with antibiotics before revealing the PCR result; the PCR test was positive in 9 out of 12. Revealing PCR results did not alter antibiotic treatment. In the 7 DIF-negative patients not given antibiotics, the PCR test was positive. In conclusion, in contrast to their responses to our multicenter paper case study, in real-life PICU physicians did not let their antibiotic prescription be influenced by real-time PCR for respiratory viruses in young children with LRTI.

In **chapter 7** real-time PCR is used to prospectively study the frequency of RSV transmission at the PICU. Transmission of RSV from patients with RSV LRTI to patients admitted to the unit for other reasons than LRTI was evaluated. Twenty-four children with RSV LRTI were admitted during the study period (total days of potential transmission: 239). Forty-eight RSV-negative patients were followed up for RSV acquisition every 5 days (total days of exposure: 683). No single RSV transmission was documented. Therefore, routine infection control measures of LRTI patients seem to be adequate to prevent RSV transmission at the PICU.

Pathofysiology of RSV

Chapter 8 evaluated the different steps in the RNA quantification process of real-time PCR for RSV. The variation of RSV RNA load in one single sample [1], the variation in two samples during one day [2], and the variation between the upper and the lower airways is reported [3]. Thirty-two infants with RSV at the PICU were included. Undiluted nasopharyngeal aspirates (NPAs) and tracheal aspirates (TAs) were taken three times a week during ventilation. Intra-sample variation [1] was shown to be minimal (<2 Cycle threshold [Ct]). Intra-day variation [2] was lowest in samples with high viral loads (95% limits of agreement -3.6 to 4.8 Ct; n=100 sample-pairs from 20 patients). RSV loads in NPAs and TAs [3], were found to be most comparable only during the early phase of infection (95% limits of agreement -5.2 to +5.8 Ct; n=31 sample-pairs from 31 patients). In conclusion, quantitative detection of RSV RNA in undiluted mucus is a reliable method to quantify viral loads, when searching for differences in the order of magnitude of 5 Ct values (≈ 30-fold changes).

Finally, in **chapter 9**, quantitative RSV real-time PCR is used to study the kinetics of disease severity, viral load, and innate and adaptive immune activation in infants with RSV LRTI at the PICU. It is controversial whether the severe symptoms of RSV LRTI of patients at the PICU is a consequence of virus induced lung pathology or whether it follows from an excessive immune responses. Furthermore, detailed knowledge on the development of primary T cell responses to viral infections in infants in general is lacking. Therefore, the dynamics of neutrophil and adaptive T cell responses in relation to viral load and parameters of disease were evaluated. Analysis of primary T cell responses in peripheral blood indicated that a substantial CD8+ T cell activation was achieved which peaked between 11-15 days after onset of first symptoms. The peak in T cell activation was preceded by a strong influx of bone marrow derived neutrophil precursors into the peripheral blood, which might reflect a compensatory mechanism for the strong influx of neutrophils into the airways, a characteristic feature during RSV infections. Upon hospital admission viral load dropped and disease severity diminished, well before T cell responses had fully developed. From the sequence of events we conclude that CD8+ T cells responses initiated during primary RSV infections are unlikely to contribute to disease when it is most severe. A causal role for RSV is more likely , since viral load was high upon admission an decreased parallel to resolution of symptoms. However, a correlation between the magnitude of viral load and disease severity in PICU patients could not be found. To explain why some patients at the PICU had to stay longer than others, additional studies need to be performed.

Finally, **chapter 10** discusses the results of the above described studies, and **chapter 11** provides an English and Dutch summary, respectively.

Nederlandse samenvatting

Bij zuigelingen en jonge kinderen komen lagere luchtweginfecties (LLWIs) vaak voor. LLWIs kunnen worden onderverdeeld in twee syndromen: bronchiolitis en longontsteking. Van vroeger uit wordt bronchiolitis geassocieerd met een virale verwekker en longontsteking met een bacteriële verwekker. Men vermoedt dat bij jonge kinderen de meeste LLWIs veroorzaakt worden door virussen. Echter, op het klinische beeld alleen kan het onderscheid tussen een virale en een bacteriële oorzaak niet worden gemaakt. Om erachter te komen of (en zo ja welk) virus de oorzaak is, worden sinds lange tijd twee soorten traditionele testen gebruikt: virale kweek directe en immuunfluorescentie testen (DIF testen). Recent is daar een derde testmogelijkheid bijgekomen: real-time PCR. Dit is een moleculaire techniek waarmee het erfelijk materiaal van een virus kan worden aangetoond. Met real-time PCR kan gevoeliger worden getest en een groter aantal luchtwegvirussen worden gedetecteerd. Dit proefschrift beschrijft het onderzoek naar de klinische implicaties van real-time PCR als routinetest in de diagnostiek van kinderen met een normale afweer opgenomen op de kinder-intensive care (IC) met een LLWI. Allereerst werd onderzocht hoe vaak verschillende virussen gevonden werden bij kinderen op de IC met het gebruik van traditionele testen (virale kweek en DIF) en met real-time PCR. Als tweede werd gekeken naar de invloed van real-time PCR testen, vergeleken met DIF, op het antibiotica gebruik bij kinderen met LLWIs op de IC. Tot slot werd onderzocht of real-time PCR betrouwbaar gebruikt kan worden als test op snotmonsters om meer inzicht te krijgen op de oorzakelijke rol van het Respiratoir Syncytieel (RS) virus bij patiënten met een LLWI.

Detectie van luchtwegvirussen

Hoofdstuk 2 behandelt ons onderzoek naar de gevoeligheid en de specificiteit van traditionele testen voor luchtwegvirussen in vergelijking met de nieuwe real-time PCR testen wat betreft het RS virus, het influenzavirus, het parainfluenzavirus en het adenovirus. Er werd gebruik gemaakt van 267 snotmonsters van in het ziekenhuis opgenomen kinderen en volwassenen met luchtwegklachten. Het gebruik van real-time PCR ten opzichte van traditionele testen verhoogde het percentage patiënten met een gevonden virus bij kinderen van 24% naar 43% en bij volwassenen van 3.5% naar 36%. Bij de meeste virussen ontdekten we dat de hoeveelheid virus, de virale load, in monsters die positief waren met zowel real-time PCR als met traditionele testen hoger was dan de virale load in de monsters die alleen positief waren met real-time PCR (en negatief ofwel gemist werden met traditionele testen).
Dit wijst op een hogere gevoeligheid met real-time PCR dan met traditionele testen.
De specificiteit van traditionele testen was bijna 100%. Dat betekent dat bijna alle monsters die positief werden bevonden met traditionele testen ook zo werden bevestigd met real-time

PCR. We hebben geconcludeerd dat met real-time PCR testen vaker RS virus, influenzavirus, parainfluenzavirus en adenovirus werden gevonden dan met traditionele testen en dat real-time PCR daarmee een mogelijk voordeel biedt in het kunnen opstarten van de optimale behandeling van een patiënt.

Vervolgens (zie **hoofdstuk 3**) hebben wij gekeken naar de toegevoegde waarde van real-time PCR als aanvulling op traditionele testen bij 23 zuigelingen en kinderen onder de 5 jaar die moesten worden opgenomen op de kinder-IC met ademhalingsproblemen als gevolg van een LLWI. Met real-time PCR werd getest op RS virus, influenzavirus, parainfluenzavirus, adenovirus, rhinovirus, coronavirus en metapneumovirus. Door te kijken naar al deze verschillende luchtwegvirussen constateerden we een grote toename in het aantal gevonden virussen, ten opzichte van het aantal aangetoonde virussen met traditionele testen. Met traditionele testen werd al bij een relatief hoog percentage patiënten een virus gevonden, namelijk bij 48%, echter met real-time PCR nam dit nog enorm toe tot 96%. Twee of meer virussen werden gevonden bij 35% van de kinderen. We concludeerden dat real-time PCR, gebruikt bij kinderen met LLWIs op de IC, een gevoelige en betrouwbare test is voor het aantonen van luchtwegvirussen en dat met de invoering van deze real-time PCR testen tweemaal zo vaak virussen worden gevonden in deze groep patiënten.

Hoofdstuk 4 beschrijft de zoektocht die we verrichtten naar alle bekende gegevens over de gevoeligheid en specificiteit van traditionele testen vergeleken met moleculaire testen (PCR testen) in de huidige wetenschappelijke literatuur. We zochten in twee databases, Medline en Embase, en doorzochten de referentielijsten van relevante artikelen op studies te vinden die traditionele testen vergeleken met PCR testen voor het aantonen van luchtwegvirussen bij zuigelingen en kinderen met luchtwegklachten. Drieëndertig publicaties (10.297 patiënten) werden geanalyseerd en het werd duidelijk dat het soort test (zowel traditioneel als PCR) per onderzoek sterk uiteen liep. Doordat de soort test per studie sterk verschilde, konden de resultaten van de studies niet direct vergeleken worden. In de verschillende publicaties liep de gevoeligheid van traditionele testen ten opzichte van PCR uiteen van 0.00-1.00 (van slecht tot uitstekend). Hierdoor was het niet mogelijk om een definitieve conclusie te trekken over de gevoeligheid van traditionele testen ten opzichte van PCR testen. Het is daarom van groot belang om internationale standaarden te ontwikkelen waaraan virale testen moeten voldoen. Alleen dan kan verzekerd worden dat elk instituut wereldwijd testmethoden gebruikt met voldoende gevoeligheid.

Tot slot, zoals beschreven in **hoofdstuk 5**, werd real-time PCR gebruikt om te kijken hoe vaak drie recent ontdekte virussen, bocavirus (2005), KI virus (2007) en WU virus (2007), voorkomen op de kinder-IC. Patiënten onder de 5 jaar met een LLWI op de IC (78 totaal) en controlepatiënten op de kinder-IC zonder een LLWI (83 totaal) werden onderzocht. Bocavirus,

KI en WU virus kwamen voor bij respectievelijk 5.1%, 0% en 2.6% in de LLWI groep en in 2.8%, 4.8% en 2.4% in de controle groep zonder LLWI. Bijna alle kinderen met boca, KI of WU virus in de LLWI groep hadden naast dit virus ook nog een ander luchtwegvirus erbij dat de klachten van de LLWI zou kunnen verklaren. Het is dus niet zeker dat de gevonden boca, KI of WU virussen ook echt oorzakelijk in verband staan met de klachten van de LLWI. Echter, er was één kind met een bocavirus dat niet een ander luchtwegvirus er nog bij had. Bij dit ene kind werd het bocavirus in een grote hoeveelheid gevonden. Hier was het gevonden bocavirus dus waarschijnlijk wel de oorzaak van de LLWI. Het was interessant om te zien dat de hoeveelheid boca, KI of WU virus in de LLWI groep groter was dan de hoeveelheid virusdeeltjes in de groep zonder LLWI. Gezien het lage totaal aantal positieve patiënten was dit statistisch niet significant. Echter, het zou wel een aanwijzing kunnen zijn dat boca, KI en WU virus een oorzakelijke rol spelen bij LLWIs als ze gevonden worden in een grote hoeveelheid, en dat ze een toevalsbevinding zijn wanneer een kleine hoeveelheid (oftewel een lage virale load) wordt aangetroffen. Kortom, boca, KI en WU virus komen niet vaak voor op de kinder-IC, niet bij kinderen mét en niet bij kinderen zonder LLWIs. Het is daarom niet waarschijnlijk dat boca, KI en WU virus belangrijke verwekkers zijn van LLWIs die leiden tot opname op de kinder-IC. Verdere studies zijn nodig om te bepalen of de hoeveelheid aanwezig virus gebruikt kan worden om onderscheid te maken tussen een echte infectie met boca, KI en/of WU en een toevalsbevinding van deze virussen.

Invloed op de behandeling van de patiënt

Hoofdstuk 6 van dit proefschrift behandelt ons onderzoek naar de invloed van het doen van real-time PCR testen voor luchtwegvirussen, als aanvulling op het gebruik van de DIF test, op het antibioticagebruik bij IC-kinderen met LLWIs. Eerst werd voor dit doel een theoretische casusstudie verricht en vervolgens werd een onderzoek in de dagelijkse praktijk gedaan. In de praktijkstudie werden kinderen onder de 5 jaar onderzocht; zij werden getest met DIF en PCR. Als bij deze kinderen een luchtwegvirus werd gevonden met de DIF test werd dit gerapporteerd op de eerste werkdag na opname. Op de tweede dag na opname gaven de artsen van de kinderen aan of ze wel of niet waren gestart met antibiotische behandeling. Vervolgens werd de uitslag van de PCR test bekend gemaakt. Na de PCR uitslag werd gekeken of de artsen het antibioticabeleid bij de patiënten veranderden of niet. Met de theoretische casusstudie (94 IC artsen in Nederland en Duitsland vulden de enquête in) werd gevonden dat indien PCR een virus aantoonde, de antibiotica bij de theoretische patiënten meestal gestopt zou worden; dit resultaat was zeer significant. In de praktijkstudie includeerden we 38 kinderen, waarvan bij 19 kinderen een virus werd gevonden met de DIF test. Van de 19 kinderen waarbij geen virus werd gevonden met de DIF test kregen 12 kinderen antibiotica voorgeschreven. De PCR test toonde bij 9 van deze 12 patiënten wel een virus aan. Het bekend maken van dit PCR resultaat

leidde er echter niet toe dat bij deze patiënten het toedienen van antibiotica werd gestopt. Bij de andere 7 DIF-negatieve patiënten die geen antibiotica kregen voorgeschreven, werd met de PCR test bij allemaal wel een virus gevonden. De PCR bevestigde dus het vermoeden van de arts dat de patiënt een virus zou hebben (daarom werd geen antibioticum gegeven). De conclusie was dat, in tegenstelling tot de resultaten van onze theoretische studie in Nederland en Duitsland, in de dagelijkse praktijk het invoeren van real-time PCR testen voor luchtwegvirussen niet leidt tot een verandering in antibioticagebruik bij kinderen met een LLWI op de IC.

Ook, zoals aan de orde komt in **hoofdstuk 7**, onderzochten we met real-time PCR hoe vaak het voorkomt dat het RS virus overgedragen wordt van het ene naar het andere kind op de IC. Hiervoor richtten we ons op kinderen met een LLWI veroorzaakt door het RS virus en we keken of deze kinderen het RS virus overdroegen op kinderen die voor een andere reden dan LLWI waren opgenomen (bijvoorbeeld voor hartchirurgie). Gedurende de studieperiode werden 24 kinderen opgenomen met een LLWI veroorzaakt door het RS virus (totaal aantal onderzochte dagen dat de kinderen het RS virus konden overdragen: 239). Bij 48 kinderen die opgenomen waren op de IC zonder het RS virus werd elke 5 dagen getest of ze het RS virus opliepen (totaal aantal onderzochte dagen dat kinderen het virus konden oplopen: 683). Geen enkel geval van RS overdracht werd vastgesteld. Blijkbaar zijn de beschermende maatregelen die worden genomen bij kinderen met LLWIs goed genoeg om te voorkomen dat RS wordt overgedragen naar medepatiënten op de IC.

Oorzakelijke rol van het RS virus

Hoofdstuk 8 beschrijft ons onderzoek naar de betrouwbaarheid van verschillende stappen in het real-time PCR proces als het gaat om het aantonen van de hoeveelheid virus in snot van een patiënt. We onderzochten [1] de variatie in de hoeveelheid virus die je vindt binnen één monster; [2] de variatie in de hoeveelheid virus die je vindt in twee verschillende samples die afgenomen zijn bij dezelfde patiënt op dezelfde dag en [3] de variatie in de hoeveelheid virus gevonden in de hogere en de lagere luchtwegen bij dezelfde patiënt op dezelfde tijd.
In totaal werden 32 zuigelingen op de IC met het RS virus onderzocht. Onverdunde snotmonsters uit de neus (hogere luchtweg) en de luchtpijp (lagere luchtweg) werden driemaal per week afgenomen zolang patiënten beademd werden. De resultaten waren als volgt. [1] De variatie binnen 1 sample was minimaal (<2 Ct waardes). [2] De variatie in de hoeveelheid virus over de dag was het kleinst in monsters met een grote hoeveelheid virus (95% van de verschillen lagen tussen de -3.6 en de 4.8 Ct waardes; 100 monsterparen werden onderzocht van 20 patiënten). [3] De hoeveelheid RS virus in de neus en de luchtpijp kwam het meest overeen gedurende de eerste paar dagen van de RS infectie (95% van de verschillen lagen tussen de -5.2

en de 5.8 Ct waardes; 31 monsterparen werden onderzocht van 31 patiënten).
Kortom, het bepalen met real-time PCR van de in onverdund snot aanwezige hoeveelheid RS virus is betrouwbaar als men op zoek is naar verschillen in de orde van grootte van 5 Ct waardes (dit komt overeen met een verschil in hoeveelheid van 30x zo klein of groot).

Tot op heden is het onduidelijk of de ernstige symptomen die zuigelingen kunnen krijgen van een LLWI met het RS virus worden veroorzaakt door het virus zelf, of dat ze worden veroorzaakt door een overijverig afweersysteem van de patiënt. Ook is het nog niet bekend hoe de verworven afweeractiviteit, de T cel reactie, bij zuigelingen precies verloopt. Daarom deden wij, zoals beschreven in **hoofdstuk 9**, onderzoek naar het verloop van de aangeboren afweeractiviteit (neutrofiel respons) en de verworven afweerreactie (T cel reactie) in relatie tot de ziekte-ernst en het verloop van de hoeveelheid RS virus bij de LLWI patiënten op de kinder-IC. Analyse van de T cel reactie op het ontdekken van een RS virus in het lichaam liet zien dat deze behoorlijk groot was 11-15 dagen na het ontstaan van de eerste luchtwegklachten.
Deze T cel reactie werd vooraf gegaan door een toevlucht van neutrofielen uit het beenmerg naar de bloedsomloop toe. Het zou goed kunnen dat deze toevlucht ontstaat als reactie op een toevlucht van neutrofielen van de bloedsomloop naar de longen, die vanuit het beenmerg wordt aangevuld. Wij zagen dat de ziekte-ernst en de hoeveelheid virus maximaal waren bij opname van de patiënt op de IC, dus helemaal in het begin van het ziekteverloop. De ziekte-ernst en de hoeveelheid virus bij de patiënt waren al flink afgenomen tegen de tijd dat de T cel reactie in het bloed was ontstaan. Uit deze volgorde blijkt dat het onwaarschijnlijk is dat T cellen bijdragen aan de ernst van de symptomen (daarvoor komen ze te laat). Het is waarschijnlijker dat het virus zelf een directe rol heeft in het ontstaan van klachten, aangezien de hoeveelheid virus hoog was bij opname en afnam parallel aan de afname van de klachten. Echter, wij vonden geen directe relatie tussen de hoeveelheid virus bij de patiënten en de ernst van de symptomen.
Verder onderzoek is nodig om te verklaren waarom sommige patiënten met LLWIs door RS lang ziek zijn en andere sneller beter worden.

In **hoofdstuk 10** werden de resultaten van de bovenstaande studies bediscussieerd.
We concludeerden dat moleculaire technieken zoals real-time PCR gevoelig en betrouwbaar zijn om te onderzoeken hoe vaak virussen voorkomen bij kinderen met een LLWI opgenomen op de IC. Met real-time PCR werd bij nagenoeg alle kinderen een luchtwegvirus gevonden, dit was tweemaal zo vaak als met traditionele testen. Echter, de implementatie van real-time PCR als standaard test op de kinder-IC in aanvulling op traditionele testen leidde niet tot een verandering in het antibioticagebruik bij IC kinderen met een LLWI. Daarom is er op dit moment geen noodzaak om real-time PCR in te voeren op de kinder-IC. Dit kan in de toekomst echter veranderen. Wellicht komen betrouwbare PCR testen ook beschikbaar voor bacteriën, waarmee een bacteriële verwekker kan worden uitgesloten. Bovendien wordt PCR mogelijk waardevol bij het behandelen van nieuwe virussen en bij het beperken van uitbraken van nieuwe virussen.

En tot slot is het belangrijk om vanuit wetenschappelijk perspectief real-time PCR verder te ontwikkelen zodat kwantitatieve bepalingsmethoden gebruikt kunnen worden om meer inzicht te krijgen in de oorzakelijke rol van luchtwegvirussen.

Tot slot werd in **hoofdstuk 11** respectievelijk een Engelse en Nederlandse samenvatting gegeven van de resultaten.

Affiliations

Grada M. van Bleek: *Department of Pediatrics, The Wilhelmina Children's Hospital, University Medical Center Utrecht, Utrecht*
Frank E.J. Coenjaerts: *Department of Virology, University Medical Center Utrecht, Utrecht*
Cornelis K. van der Ent: *Department of Pediatric Pulmonology, Wilhelmina Children's Hospital, University Medical Center Utrecht, Utrecht*
Johannes Forster: *Department of Pediatrics, St. Josefskrankenhaus and University Hospital Freiburg, Freiburg*
Nicolaas J. G. Jansen: *Department of Pediatric Intensive Care, Wilhelmina Children's Hospital, University Medical Center Utrecht, Utrecht*
Vera M. Kamp: *Department of Respiratory Medicine, University Medical Center Utrecht, Utrecht*
Jan L. L. Kimpen: *Department of Pediatric Infectious Diseases, Wilhelmina Children's Hospital, and Executive Board, University Medical Center Utrecht, Utrecht*
Els Klein Breteler: *Department of Virology, University Medical Center Utrecht, Utrecht*
Leo Koenderman: *Department of Respiratory Medicine, University Medical Center Utrecht, Utrecht*
Anton M. van Loon: D*epartment of Virology, University Medical Center Utrecht, Utrecht*
Michaël V. Lukens: *Department of Pediatrics, The Wilhelmina Children's Hospital, University Medical Center Utrecht, Utrecht*
Monique Nijhuis: *Department of Virology, University Medical Center Utrecht, Utrecht*
John W. A. Rossen: *Department of Virology, University Medical Center Utrecht, Utrecht, and Laboratory of Medical Microbiology and Immunology, St. Elisabeth Hospital Tilburg, Tilburg*
Maroeska Rovers: *Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht, Utrecht*
Rob Schuurman: *Department of Virology, University Medical Center Utrecht, Utrecht*
Carline E. A. Tacke: *Department of Pediatric Infectious Diseases, Wilhelmina Children's Hospital, University Medical Center Utrecht, Utrecht, and Department of Pediatric Hematology, Emma Children's Hospital, Academic Medical Center, Amsterdam*
Cuno S.P. Uiterwaal: *Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht, Utrecht*
Laurien H. Ulfman: *Department of Respiratory Medicine, University Medical Center Utrecht, Utrecht*
Marco C. Viveen: *Department of Virology, University Medical Center Utrecht, Utrecht*
Tom F. W. Wolfs: *Department of Pediatric Infectious Diseases, Wilhelmina Children's Hospital, University Medical Center Utrecht, Utrecht*
Marieke M. van der Zalm: *Department of Pediatric Pulmonology, Wilhelmina Children's Hospital, University Medical Center Utrecht, Utrecht*

List of publication

This thesis

(1) Van de Pol AC, Rossen JW, Wolfs TF, Klein Breteler EK, Kimpen JL, van Loon AM, Jansen NJ. Transmission of respiratory syncytial virus at the paediatric intensive care unit: a prospective study using real-time PCR. *Clin Microbiol Infect 2009 (in press)*

(2) Van de Pol AC, Wolfs TF, Jansen NJ, Kimpen JL, van Loon AM, Rossen JW. Human bocavirus and KI/WU polyomaviruses in pediatric intensive care patients. *Emerg Infect Dis 2009;15:454-7.*

(3) Van de Pol AC, van Loon AM, Wolfs TF, Jansen NJ, Nijhuis M, Breteler EK, Schuurman R, Rossen JW. Increased detection of respiratory syncytial virus, influenza viruses, parainfluenza viruses, and adenoviruses with real-time PCR in samples from patients with respiratory symptoms. *J Clin Microbiol 2007;45:2260-2.*

(4) Van de Pol AC, Wolfs TF, Jansen NJ, van Loon AM, Rossen JW. Diagnostic value of real-time polymerase chain reaction to detect viruses in young children admitted to the PICU with lower respiratory tract infection. *Critical Care 2006;10:R61*

Other

(1) Schouten ES, van de Pol AC, Schouten AN, Turner NM, Jansen NJ, Bollen CW. The effect of aprotinin, tranexamic acid and aminocaproic acid on blood loss and use of blood products in major pediatric surgery: a meta-analysis. *Pediatr Crit Care Med 2009;10:182-90.*

(2) Murphy CG, van de Pol AC, Harper MB, Bachur RG. Clinical predictors of occult pneumonia in the febrile child. *Acad Emerg Med 2007;14:243-9.*

(3) ten Cate O, ter Braak EW, Frenkel J, van de Pol AC. De 2-tot-10 verwacht niveau-schaal (2-10VN-schaal) bij persoonlijke beoordelingen. *Tijdschrift voor Medisch Onderwijs 2006;25:157-163.*

(4) Van de Pol AC. Boekverslag: Anatomie van het gevoel, dagboek van een co-assistent. *Tijdschrift voor Medisch Onderwijs 2004;23:202.*

Curriculum vitae

Personalia

Naam:	Alma Cécile van de Pol
Geboren:	16-03-1980 te Eindhoven
Burgerlijke staat:	Samenwonend, 1 kind

Opleiding

2006 - heden	**Universiteit Utrecht** Promotietraject; Alexandre Suerman MD-PhD programma, UMC Utrecht. Titel: "Moleculaire detectie van respiratoire virussen: klinische implicaties "Co-promotoren: Dr. T.F.W. Wolfs, Dr. N.J.G. Jansen, Dr. J.W.A. Rossen Promotor: Prof. dr. J.L.L. Kimpen *Divisie Kinderen, Wilhelmina Kinderziekenhuis, UMC Utrecht* *Afdeling Virologie, UMC Utrecht*
1999 - 2006	Geneeskunde studie (afstudeerdatum 30 januari 2006)
2006 - 2009	**Erasmus universiteit Rotterdam** Master Clinical Epidemiology (afstudeerdatum 28 augustus 2009)
2001 - 2003	**Utrecht school of governance/economics** Extra curriculaire vakken Internationale Samenwerking en Economie (20 weken)
1993 - 1999	**Van Maerlant lyceum Eindhoven** VWO diploma cum laude in 9 vakken
1996 - 1997	**Collegio de bellas artes, Maracaibo, Venezuela** Uitwisselingsjaar met de American Field Service (AFS)

Werkervaring

heden	**Sint Antonius ziekenhuis, Nieuwegein** Werkzaam als arts-assistent op de afdeling kindergeneeskunde
2005 – 2006	**Wilhelmina kinderziekenhuis, utrecht** Afstudeerstage kindergeneeskunde, schakeljaar • Algemene semi-arts stage (ASAS) op de afdeling Kinder-IC • Wetenschappelijke stage bij de afdeling kinder-infectieziekten Begeleiders: Dr. T.F.W. Wolfs, Dr. N.J.G. Jansen, Dr. J.W.A. Rossen
2004	**Harvard university, Boston, USA** Wetenschappelijke stage kindergeneeskunde "Clinical predictors of occult pneumonia in the febrile child" *Division of Emergency Medicine, Children's Hospital Boston, Massachusetts*
2003	Keuze co-assistentschap keel- neus- en oorheelkunde *Department of Otorhinolaryngology, Massachusetts Eye and Ear Infirmary*
2002	**"Sadhana village", Puna, India** • Ontwikkelingswerk, internationale studentenvereniging AIESEC, activiteitenbegeleiding in opvangtehuis voor mensen met een verstandelijke beperking • Stageverslag "Geneeskunde voor patiënten met een verstandelijke handicap" *Medische psychologie, UMC Utrecht*

Beurzen

- European Society of Clinical Microbiology and Infection Diseases Research Grant, 2008
- Alexandre Suerman MD-PhD programma UMC Utrecht, 2006
- K.F. Hein studie fonds en Trajectumbeurs Universiteit Utrecht, 2004

Dankwoord

Mijn dank aan de volgende mensen is groot:

Kinderen die meededen aan het onderzoek en hun ouders: Hartelijk dank voor jullie deelname, tijd en vertrouwen.

Co-promotoren – Tom Wolfs, Koos Jansen, John Rossen: Ik kan mij geen betere co-promotoren wensen. Bedankt dat jullie ondanks mijn eigenwijsheid, ongeduld, zwangerschap, drang naar controle etc. de moed niet opgaven ☺. Met veel plezier kijk ik terug op onze +/- 150 onderzoeksbesprekingen op de vrijdag. Ik heb grote waardering voor jullie gedegen begeleiding en overweldigende betrokkenheid, waardoor ik nu terugkijk op 3 mooie jaren.

Promotor – Jan Kimpen: Bedankt dat je vanaf het begin bij mijn onderzoek betrokken was. Het was fijn dat je de voortgang in de gaten hield. Ongelofelijk dat je tijd voor mij wist vrij te maken als dat nodig was.

Kinder-IC WKZ – Hans van Vught, Sjef van Gestel, Casper Bollen, Esther Veldhoen, Annemieke Miedema, Marije Hennus, Fanny Wigman, Eric Veltman, Alvin Schadenberg, Selma Agra, Liseth de Kock, Jeannette Slagmolen, Antje Boessenkool, Joke de Wilde en alle verpleegkundigen die winters lang snotjes hebben gespaard: Bedankt voor de fantastische tijd. Ik mis jullie!

RSV onderzoeksgroep WKZ – Louis Bont, Marieke Ermers, Eltje Bloemen, Michiel Houben, Beatrijs Bloemers, Tina Faber, Mirjam Belderbos, Annemieke Schuurhof, Maarten Blanken, Marij van Helden, Loes Nibbelke, Pauline Winkler, Michaël Lukens, Grada van Bleek: Bedankt dat jullie me wilden “adopteren”, dank zij jullie voelde ik me van meet af aan thuis in WKZ onderzoeksland. Louis, bedankt dat ik mee mocht met de leuke uitjes die je organiseerde. En behalve dat het heel gezellig was, zorgden de meer dan wekelijkse RSV besprekingen ook voor de nodige verdieping van mijn kennis die ik goed kon gebruiken.

Arts-onderzoekers WKZ – Marieke van der Zalm, Sanne Nijhof, Titia Niers, Ellen Wehrens, Hubert van der Doef, Hannelie Engbers, Marije Koopman, Samantha Gouw, Marieke van Summeren, Berber Kapitein, Anne de Pagter, Judith Sittig, Joost Aalberse, Pauline van Leeuwen, Anne van Gugten en natuurlijk paranimfen Martijn Slieker en Gerdien Tramper: Bedankt voor de kopjes koffie, de adviezen, het luisterende oor, de praktische info, het lunchen, nog meer kopjes koffie. Ik heb geboft met zulke leuke collega’s!

Virologie UMC Utrecht – Ton van Loon, Frank Coenjaerts, Rob Schuurman, Annemarie van het Veen, Els Klein-Breteler, Ernst-Jan Jacobsen, Stefanie Zuurveen, Machiel Vos, Lydia Tan, Barry Bennaissa-Trouw, Henny KatsRenaud, en zovele anderen: Bedankt voor het meedenken met mijn onderzoek en de praktische hulp in het lab. Dankzij jullie weet ik nu dat ik labwerk niet alleen interessant vind maar ook leuk en gezellig. Wie had dat kunnen denken?

Studenten – Esther Schouten, Bart Waalewijn, Niels Lastdrager, Carline Tacke en natuurlijk Marco Viveen: Ik kan jullie niet genoeg bedanken voor de bergen werk die jullie verzet hebben. Esther en Bart, het was heel bijzonder om mijn onderzoek tijdens mijn verlof met zoveel vertrouwen in jullie handen te kunnen geven. Niels, bedankt voor je grote inzet. Carline, je hebt me overdonderd met je werkwijze en wat leuk dat we collega's zijn geworden. Marco, zonder jou was het nooit gelukt. Als iemand vakantie verdient, ben jij het.

Kindergeneeskunde St Antonius Ziekenhuis Nieuwegein: collega arts-assistenten, kinderartsen, en alle andere medewerkers: gelukkig was dit boekje bijna af toen ik bij jullie kwam! Bedankt voor jullie hulp bij het "inkomen" in de kliniek, het was een hele bijzondere tijd om mee te maken.

Richard Bachur, Charlie Murphy, Pat Cox, Graham Colditz, Josiah Seale and family, Mulligans: Thank you for having me in Boston, thank you for the opportunity to find out that I like doing research a lot.

Maaik, An, Charlie, Luuk, Natas, Anne, Loes, Brech, Suus + mannen/kinderen, P&A en Fiene en Lily, M&M en Daan: Bedankt dat ik altijd op jullie kan rekenen.

Eduardo/Aura/Danny/Gaby/Santiago/Paula/Kristel/Virginia/Roberto/Mario: Este librito es! Vamos a ver si les llegue por correo... Quidense, les quiero mucho.

Papa/mama/Eva/Carlos/Iris/Thomas/Ahmed/Maaike/Mèrghani/IJda/Beppe/Sjoerd/Emilie/Mare en tot slot Jaap en Fenna: Aan jullie heb ik alles te danken. Liefs!