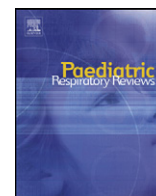




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Review

New molecular virus detection methods and their clinical value in lower respiratory tract infections in children[☆]Tuomas Jartti^{1,*}, Maria Söderlund-Venermo², Klaus Hedman², Olli Ruuskanen¹, Mika J. Mäkelä³¹ Department of Pediatrics, Turku University Hospital, Turku² Department of Virology, University of Helsinki, Helsinki³ Pediatric division, Department of Allergy, Skin and Allergy Hospital, University of Helsinki, Helsinki, all in Finland

EDUCATIONAL AIMS

- To give an overview of the modern respiratory virus PCR assays
- To review the applicability of these methods to lower respiratory tract illnesses in children
- To help physicians to use and interpret respiratory virus PCR assays optimally in their clinical practice

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SUMMARY

During the past decade, several new respiratory viruses and their subgroups have been discovered. All these new viruses, as well as previously known respiratory viruses, can be detected by sensitive PCR methods, which have become popular in the diagnostic workup of respiratory viral infections. Currently, respiratory viruses can be detected in up to 95% of children with lower respiratory tract illness. On the other hand, virus detection rates in asymptomatic children are also high (up to 68%), as are coinfection rates in symptomatic children (up to 43%) and justified concerns of causality have been raised. Imposing progress has been made in developing multiplex quantitative PCR assays; here, several primer sets are run within a single PCR mixture. These PCR assays give a better understanding of the dominant viral infection, of viral infections that may be incipient and of any waning infections than does a single-target PCR. Multiplex PCR assays are also gaining popularity due to their cost-effectiveness and short throughput time compared to multiple single-target PCRs. Our understanding of the indications of virus PCRs and our ability to interpret the results from a clinical point of view have improved. This paper reviews the progress in PCR assays and discusses their role in the diagnosis of lower respiratory tract infections in children.

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INTRODUCTION

The diagnostics of respiratory viral infections began in 1933 by the discovery of the influenza A virus.¹ Over the next three decades, several other major respiratory viruses were discovered, including enteroviruses, the adenovirus, the respiratory syncytial virus

(RSV), the rhinovirus (HRV), the parainfluenza virus (PIV), and the coronavirus. The detection methods consisted of virus culture, antigen detection and serology. These methods yielded an overall virus detection rate of only less than 40% in children with lower respiratory illnesses (acute wheezing/asthma and pneumonia).^{2,3}

In the 1990s, a new era began with the development of molecular amplification-based techniques (PCR, polymerase chain reaction). Not only did PCR revolutionize the detection sensitivity of known viruses but it also enabled the discovery of altogether new respiratory viruses in the respiratory tract, including the human metapneumovirus (HMPV), new coronaviruses, the human bocavirus (HBoV), HRV-C group and polyomaviruses.⁴ Molecular methods detect currently one or more respiratory viruses in up to 95% of children with bronchiolitis, acute wheezing or asthma, and in up to 72% of children with pneumonia (Table 1).^{5–10} PCR has achieved wide use in clinical virology, and is displacing the conventional methods with the exception of commercial rapid antigen detection tests for influenza virus and RSV. The high

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* Corresponding author. Department of Pediatrics, Turku University Hospital, P.O. Box 52, 20520 Turku, Finland. Fax: +358 2 313 1460.

E-mail address: tuomas.jartti@utu.fi (T. Jartti).

Abbreviations: DNA, deoxyribonucleic acid; HBoV, human bocavirus; HMPV, human metapneumovirus; HRV, human rhinovirus; LRTI, lower respiratory tract illness; n, number of samples; NP, nasopharyngeal; NPA, nasopharyngeal aspirate; PCR, polymerase chain reaction; PIV, parainfluenza virus; qPCR, quantitative PCR; RNA, ribonucleic acid; RSV, respiratory syncytial virus; RT, reverse transcriptase; RVP, respiratory virus panel.

Table 1

Virus etiology of lower respiratory illnesses in children.

Illness	Dominant virus	Virus coinfection rate	Total detection rate
Bronchiolitis	RSV	41%	95%
Acute wheezing/asthma	HRV	43%	95%
Pneumonia <12 months,	RSV, HMPV	33%	77%
>12 months	HRV, HBoV	27%	72%

RSV, respiratory syncytial virus; HRV, human rhinovirus; HMPV, human metapneumovirus; HBoV, human bocavirus.^{5–10}**Table 2**

Advantages and disadvantages of PCR-based assays.

Method	Advantages	Disadvantages
PCR-based vs. conventional methods	<ul style="list-style-type: none"> • superior sensitivity and specificity • sample type and handling not as critical; the viruses do not need to be infectious, no need for intact cells • easy to automate with current technology • can be used to both detect and quantify the specific virus • can be used as multiplex, enabling detection of several agents in one tube • product can be genotyped • product can be sequenced, enabling molecular epidemiology • provides an early diagnosis; often positive before serology 	<ul style="list-style-type: none"> • false negatives due to inhibition of the polymerase, or other reaction failures • false positives due to contamination • expensive • require proper facilities, instrumentation and procedures • require purification of nucleic acids before the test • require many positive and negative controls • may be clinically even too sensitive, the result needs cautious interpretation • normally, only the virus that is looked for can be found (≠culture) • cannot detect past infections • or distinguish primary from secondary, or acute from chronic infections (≠serology)
Nested PCR vs. normal end-point PCR	<ul style="list-style-type: none"> • even more sensitive • does not necessarily need downstream hybridization • more specific than end-point PCR without hybridization 	<ul style="list-style-type: none"> • more prone to contamination and needs further precautions • more expensive • takes twice the time • requires 2 separate machines with separate facilities
Real-time (non-quantitative) PCR vs. end-point PCR	<ul style="list-style-type: none"> • can be highly automated, user friendly with less hands-on time • is faster, has higher precision, resolution and lower variability • allows real-time measurement • can be designed as a probe format which does not need a downstream method for product identification (such as Southern hybridization) • can be designed to genotype the virus or to find a mutation according to the melting points of the products • is less prone to contamination due to the closed system 	<ul style="list-style-type: none"> • more expensive due to instrumentation and reagents • need a computer and sophisticated software
Real-time quantitative PCR vs. real-time non-quantitative PCR	<ul style="list-style-type: none"> • analyses virus load, either relative or exact • better evaluation of timing of infection • can be used for therapy monitoring 	<ul style="list-style-type: none"> • more difficult to design • needs a known standard dilution series • quantifying cutoffs maybe higher than detection cutoffs, lowering sensitivity
Multiplex PCR vs. singleplex PCR	<ul style="list-style-type: none"> • detects several different viruses in the same tube • better cost-effectiveness than multiple single-PCR assays (lower expense of reagents, less laborious and shorter throughput time) • less sample consuming • provides naturally several internal controls (one positive reaction increases the negative predictive value of the others), and is more effective in determining the quality of the template • can be coupled with downstream differentiating methods, such as beads or microarrays, which are multiplex technologies that allow detection of 10-2 million analytes in a single sample 	<ul style="list-style-type: none"> • more difficult to design, may need substantial redesigning and optimization • adding multiple primer and probe sets may compromise the accuracy and sensitivity • may provide more variability • due to limited fluorophores or fluorescent spectra, to distinguish more than five viruses usually an expensive downstream detection system is needed • most bead- or microarray-applying amplicon-detection assays lack quantifying properties

sensitivity of PCR has, on the other hand, shown that viruses in asymptomatic children (up to 40–68% during high prevalence seasons) and virus coinfections - or rather codetections - in symptomatic children are common (up to 43% in acute asthma).^{5,11–15} This has raised concerns not only of causality but also whether upper airway specimens really reflect the conditions in the lower airways with regard to viral replication, since the mere presence of a virus may not be etiologically related to the patient's symptoms. In this paper, we review the latest achievements in the molecular diagnostics of respiratory viral infections and discuss their applicability to lower respiratory tract illnesses (LRTI) in children.

SINGLE-TARGET PCR

The main advantage of amplification of viral genetic material by PCR is high analytical, and often also diagnostic, sensitivity (Table 2). The first PCRs were single-target endpoint assays which allowed product detection only at the endpoint of the PCR amplification. With RNA viruses, reverse-transcriptase PCR (RT-PCR) needs to be used: reverse transcriptase first converts RNA to complementary DNA (cDNA) after which ordinary PCR may be performed. The final PCR or RT-PCR product must be certified to be correct DNA, e.g. by a post-PCR hybridization with a labelled probe. Increased specificity and sensitivity can be achieved by nested PCR

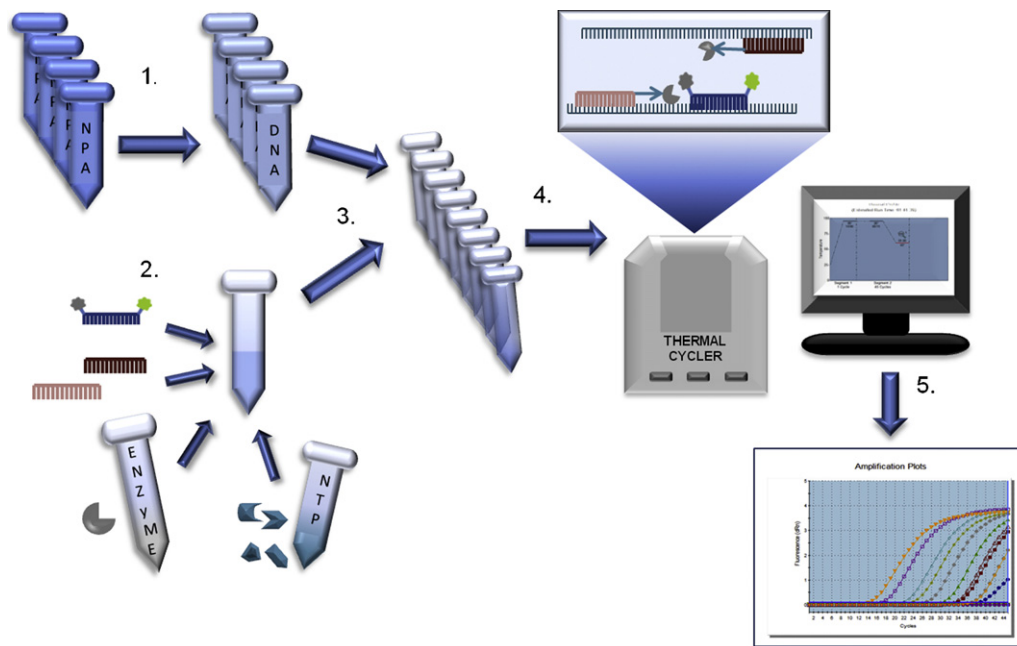


Figure 1. Overview of the real-time qPCR protocol. 1. Before PCR, the genetic material needs to be purified from the sample in a sample-preparation room. 2. In a DNA-free reaction-preparation room, water, buffer, nucleotides, primers, probe and a heat-stable polymerase enzyme are mixed and aliquoted in reaction tubes, which are taken to the sample room. 3. Purified sample DNA or RNA, standard dilutions, and controls are added to separate reaction tubes, which are then applied to the PCR instrument in a separate amplification room. 4. During PCR the carefully designed specific oligonucleotide primers (in red) are annealed to their complementary regions of the two heat-denatured single-stranded DNA molecules. Progeny strands are synthesized by extension of the primers along the full template strand by the action of the DNA polymerase. This is repeated in 30–45 temperature cycles causing an exponential amplification of the target DNA sequence. At each cycle, the target-specific fluorescent probe (in blue) is hybridized to the increasing amount of targets during which fluorescence is measured. 5. This increase in fluorescence is shown in real time on the screen. The fluorescence curves of the samples are analyzed by the computer and compared with those of the known standard, and the quantity of DNA is calculated. NPA, nasopharyngeal aspirate sample; NTP, deoxyribonucleoside triphosphates (dNTP: dATP, dGTP, dCTP, dTTP); qPCR, quantitative polymerase chain reaction.

in which two sequential pairs of primers are used instead of one (Table 2). The first or outer pair yields a DNA fragment, as is the case in a standard PCR. Then, in a subsequent PCR, a second pair of primers, called nested or inner primers, binds within the first PCR product (amplicon) to yield a shorter DNA fragment. This makes the PCR more sensitive but also more prone to contamination. Moreover, were a non-specific amplicon created in the first round, it would not be amplified in the second round (i.e. increased specificity). PCRs like these result in either a “positive” or “negative” result, but the result is not quantitative.

QUANTITATIVE PCR

Quantitative PCR (qPCR) is a method that not only detects specific DNA or RNA in a sample, but also quantifies it. By modern techniques this is achieved by automatic and computerized PCR devices (Figure 1). One or more fluorescent probes, specific for the target DNA, can be applied into the same reaction mixture together with the target, primers, nucleotides and polymerase; now hybridization occurs at each PCR cycle. The emitted fluorescent signals correlate with the amount of PCR product produced. The PCR device measures fluorescence once every cycle, i.e., data is retrieved online and at real time. These features make real-time qPCR more suitable for respiratory virus diagnosis than conventional end-point PCR.

MULTIPLEX PCR

Respiratory infections can be caused at least by any one of the currently known 26 respiratory viruses.⁸ It was soon realized that PCR was too laborious, expensive, and sample consuming, if a sufficient number of virus analyses was to be made to be clinically meaningful. Many children with respiratory illness turned also out

to have virus coinfections – sometimes no less than five respiratory virus species concomitantly.^{5,11,14} Since the clinical signs and symptoms of respiratory virus infections overlap and are seldom pathognomonic for any specific virus and since the sensitivity of conventional diagnostic methods is low, the need for testing for multiple viruses simultaneously by PCR became apparent.^{2,3} Multiplex PCR is defined as running more than one PCR analysis in a single reaction tube.¹⁶ This is accomplished by applying more than one set of primers that produce amplicons of varying sizes and are specific for different, individual viral sequences (Figure 1). A sign of professional primer and probe design is careful avoidance of cross- and mishybridization.

Multiplex PCR approaches are becoming increasingly popular for the detection of respiratory viruses, since lower reagent costs and shorter throughput time favour the cost-effectiveness of multiplex PCR over multiple single PCRs (Table 2).^{17–20} The current cost of a respiratory virus 13-plex PCR is 140 euros in our hands and the result is available within the same working day. Rapid results are crucial for clinical decisions.²¹ Multiplex PCR may be qualitative or quantitative.

Tagging (xTAG^R) and bead-based (xMAP^R) multiplex technologies are interesting applications of the technique; they allow for the detection of 50–500 analytes (protein or nucleic acids) in a single sample.²² After a multiplex PCR reaction, target-specific extension primers are attached to an xTAG universal tag sequence which is hybridized to a complementary anti-tag sequence coupled to a particular xMAP bead set. The hybridized beads are finally read by the Luminex system. The multiplex PCR assays have generally been much more sensitive than conventional viral diagnostics^{23–27} with specificity and sensitivity similar to those of single-target PCRs (Table 3).^{25–31} There are, however, slight virus-specific differences between multiplex PCRs and single-target PCRs. These differences seem to vary, and in some studies,

Table 3
Recent studies comparing different PCR methods in children with respiratory tract illness.

1 st author (year)	n	Age	Sample	Methods	No. of viruses tested	Main results
Roh (2008) ²⁴	50	84% <6 years	92% NPA 8% other	multiplex PCR virus culture, antigen detection	8	80% of samples showed concordant results
Kim (2009) ²³	101	mean 7 years	various	multiplex PCR virus culture	12	Concordance 89%
Raymond (2009) ²⁸	221	children	NPA	multiplex PCR multiplex PCR with primer extension and microarray hybridization	23	Concordance 94%
Gadsby (2010) ²⁵	286	median 5 years	respiratory	multiplex xTAG fast assay single and multiplex PCRs virus culture, antigen detection	15	xTAG had sensitivity of 79% and specificity of 100% compared to qPCR
Arens (2010) ²⁶	410	paediatric	mostly NP swabs	multiplex PCR virus culture, antigen detection	6	Sensitivities of multiplex PCR for individual viruses 94–100% and specificities 99–100%
Rand (2011) ²⁷	200	61% <18 years	71% upper respiratory	multiplex PCR multiplex xTAG virus culture, antigen detection	8	Sensitivities 86–100% and specificity 100% for both
Jansen (2011) ²⁹	133	median 12 months	NPA	single-target PCR multiplex qPCR	14	Good concordance
Pabbaraju (2011) ³⁰	334	43% <2 years	73% NP swabs/NPA	multiplex xTAG classic assay multiplex xTAG fast assay	12	Overall sensitivities classic 89% and fast assay 78%
Ali (2011) ³¹	225	<12 months	nasal and throat swab	multiplex PCR individual qPCR	11	Good agreement for most viruses

n, number of samples; NP, nasopharyngeal; PCR, polymerase chain reaction; qPCR, quantitative PCR; NPA, nasopharyngeal aspirates.

multiplex PCRs have been less sensitive than a single-target PCR for RSV, human metapneumovirus, enteroviruses, PIV or adenovirus.^{25,26,28,29,31}

UPPER AND LOWER AIRWAY SAMPLES

Respiratory viruses are usually identified in samples from the upper respiratory tract. All nasopharyngeal sample types, including nasopharyngeal aspirates (NPA), washes, swabs or brush samples, appear to allow for equally sensitive PCR analysis.^{32–34} What has been considered, however, is to what extent upper airway virus findings reflect true viral infections in the lower airways. Paediatric studies using bronchoalveolar lavage or lung biopsy samples have indicated that many respiratory viruses seem to penetrate the distal bronchioles and can be demonstrated at similar detection rates in upper and lower airways.^{13,35,36} Also asymptomatic children have exhibited high (35–43%) virus detection rates in lower airway samples.^{13,15,36}

It has been presumed that the most frequent causative agent of the common cold, HRV, occurs mainly in the upper airways, and that it is associated closely with exacerbations of asthma.^{5,11} Indeed, HRV has been found by *in situ* hybridization in lower bronchial biopsy specimens of 45% of young children with recurrent asthma-like symptoms.¹³ Findings of HRV also correlated with decreased airway conductance and a history of respiratory symptoms. Another study on tracheal and nasal secretions from paediatric patients with a tracheostomy demonstrated HRV in the lower airways of patients with community-acquired colds; the possibility of nasal contamination was excluded. HRV was detected by PCR both in nasal and tracheal secretions.³⁵ In agreement with this observation, HRV replicates well at lower airway temperatures *in vitro*.³⁷

PCR studies of induced sputum samples have shown that community-acquired pneumonia is associated with viral infection in up to 72% and viral or bacterial infection in up to 97% of the children affected.⁹ Although sputum induction did increase the sensitivity and specificity of diagnosis of lower airway infections, most of the pathogenic viruses were often identified also in

samples from the upper airways.³⁸ Although sputum induction does provide good quality samples from the lower airways, it is not recommended for routine use in children with LRTI since it is unpleasant and does not usually provide unique or additional virological information.

ILLNESS SEVERITY

Some studies have been performed to address the association between virus PCR positivity in upper airway samples and symptom severity. In two studies, HRV was more prevalent in children with respiratory symptoms (mainly wheezing) when adjusted for findings in asymptomatic controls.^{39,40} A study involving infants with recurrent respiratory infections showed that the total virus detection rates, the virus coinfection rates and HRV findings did correlate with symptom severity.¹⁴ Studies on young children have shown that RSV is most closely associated with severe bronchiolitis or LRTI.^{10,41,42} Of the HRV strains, the HRV-A and -C groups have been linked to more severe illness than the B group - especially in children.^{43,44} HRV-C may cause viremia more often than HRV-A and HRV-B.⁴⁵ The virus (including HRV) detection rate in samples from the lower airways of asthmatic children during asymptomatic periods has been the same as of healthy children.³⁶ The associations between illness severity and virus load or coinfection are discussed in the next sections.

VIRUS LOAD

Can qPCR improve the diagnosis of viral infections over end-point PCR? A recent study showed that children with LRTI have an increased total virus load and harbor more viruses in their NPA than children with no LRTI but with upper respiratory tract infection, fever or cough.⁴² Individual loads of HRV and PIV-2 were higher in LRTI than in non-LRTI, and qPCR yielded more viruses (including coinfections, where a “dominant virus” was typically identified) than the conventional methods, virus culture and direct fluorescence assay. Similarly, in immunocompetent paediatric patients HRV infection was most closely associated with LRTI in the

absence of other viral agents when the viral load was $>10^6$ RNA copies/mL.⁴⁶ Two previous reports could not identify a link between HRV load and respiratory symptoms in children.^{47,48} HRV probably causes asymptomatic infections more often than is generally assumed.¹⁵

RSV infection is short-lived and the causative agent occurs rarely in asymptomatic controls; hence, a positive RSV result is almost always of clinical relevance, regardless of the quantity of the virus.²⁹ However, a recent study reported a link between RSV load (but not between HBoV or HMPV loads) and the risk of *Streptococcus pneumoniae* or *Haemophilus influenzae* acute otitis media in young children.⁴⁹ In another paediatric study, the HMPV load was associated with fever, bronchodilator use, chest radiography, and duration of hospital stay, i.e. obvious markers of disease severity.⁵⁰ Studies on the H1N1 influenza A pandemic virus have shown that asymptomatic virus infections occur.^{51,52} There is also a direct association between, on the one hand, pharyngeal viral replication and host cytokine responses and, on the other hand, severity of respiratory illness. The virus load decreases markedly in response to antiviral treatment.^{53,54}

Studies on HBoV1 have consistently shown persistence or recurrence of HBoV1 DNA in the upper airways at low load long after infection. On the other hand, a high HBoV1 load is reportedly associated with acute HBoV infection and LRTI.^{55,56} Due to virus persistence, a diagnosis of acute HBoV1 infection should be primarily based on serology or serum PCR, and secondarily on qPCR with a cutoff of $>10^4$ genomes/ml of NPA.⁵⁶ It is currently not known if low HBoV loads are due to persistence of HBoV DNA, to mucosal contamination, to re-infection or reactivation of latent HBoV, or to prolonged low-level replication and shedding of HBoV, which could be related to damage of the epithelium of the airways by other viral infections or airway disease. Adenoviruses also are frequently found in respiratory coinfections at low viral loads: apparently due to persistence or latency.^{57,58} In contrast to HBoV, the adenovirus load correlates poorly with illness severity.^{55,59}

Looking at virus loads longitudinally (assessed at hospital admission, discharge and post-discharge follow-up visits) in the NPAs of young infants of the general population, a sustained decrease in the RSV load takes place.⁶⁰ Another study on children hospitalized for LRTI showed that the viral loads in the NPA decreased in samples taken 3–4 days apart. This was true for most single and viral coinfections, but not for HRV and HBoV infections.⁵⁹ There was a marked decrease in the occurrence of positive HRV cultures among adolescents with common cold from day 1 to day 7; most of the patients remained positive by RT-PCR.^{61,62} Ultimately, a rise and fall in the number of virus copies during an acute respiratory illness would be needed to demonstrate a causal relationship between virus PCR positivity and respiratory illness.⁶³

COINFECTIONS

The distinction between coinfection and codetection has been ignored in most studies, whereby we use the word coinfection in this connection. In a genuine coinfection, two viruses infect the patient at about the same time. In codetection, one or both of the viruses detected may be remnants of a past infection. By combining qPCR with serology, coinfection can be separated from codetection.

Virus-virus coinfections occur in up to 43% of children with LRTI. Such coinfections may contribute to illness severity.^{5,7,10} Interestingly, some viruses occur more often in coinfections than others, e.g. HBoV1 has been detected with other viruses in up to 78% of cases.⁵⁶ Fever, leucocytosis, and the use of antibiotics are more common among patients with RSV coinfection than among patients with an infection caused by RSV alone.⁵⁹ HRV coinfections

have been associated with illness severity in infants with recurrent respiratory infections.¹⁴ In children with pneumonia, viral coinfections have been associated with illness severity,^{6,64} but the severity of bronchiolitis was not associated with virus coinfection in one study.¹⁰

Viral–bacterial coinfection occurs in up to 66% of children with community-acquired pneumonia, and with any combination of codetections between virus and bacteria in up to 84% of the cases.^{6,8,9,65} The most frequent combinations are *Streptococcus pneumoniae* with influenza A or HRV. Virus–bacteria coinfections may cause a more severe illness than viral or bacterial infections caused by one pathogenic organism.^{66–68} Many reports have shown that mixed influenza (A or B) virus–*Staphylococcus aureus* infections may cause fatal pneumonia in children.^{67,68} Treatment failures appear to be linked to mixed viral–bacterial pneumonias in children.^{9,69} In children with invasive pneumococcal disease, viral coinfections are common (34%) and are probably associated with higher mortality.⁷⁰ In adults, there is also a link between the severity of asthma exacerbations (which are usually associated with viral infection) and the prevalence of invasive pneumococcal disease.^{71,72}

CLINICAL INTERPRETATION OF PCR

Can we rely on PCR-based respiratory virus detection alone? This question was asked often in the early days of PCR. Profound methodological improvements over the years have reduced the likelihoods of false positive and false negative results. The measures to improve the performance of PCR assays include stringent precautions to avoid contaminations, e.g. using filtered tips, separate rooms and laminar flow hoods, high-standard nucleic acid preparations kits, internal positive controls and numerous negative controls. Inadequate reporting of experimental details regarding qPCR can still impede our ability to evaluate critically the results of scientific papers and the reliability of the specific reported qPCR method. Helpful and useful guidelines are available aiming at improving the quality of reporting of new qPCR methods, e.g. the *Minimum Information for publication of Quantitative real-time PCR Experiments* (MIQE).⁷³

Clinically, the detection rates have generally been higher in symptomatic than asymptomatic individuals. The detection rates have also been higher in asymptomatic individuals with a chronic condition than in asymptomatic individuals without such a condition; this argues against a significant role for false positive results.¹⁵ Perhaps a still more important technical matter is the sensitivity of the PCR – it is generally higher than the sensitivity of conventional methods,^{23–27} and therefore, PCR is likely to detect more viruses which may occur at different phases of infections, e.g. incipient, acute and past infections. The risk of false negative findings increases as the number of primer and probe sets is increased, but this risk is ameliorated by inserting internal controls in the reaction tube in order to reveal inhibiting factors in the sample.^{18–20}

Several findings suggest that PCR is likely to detect true respiratory infections irrespective of symptoms. First, although many respiratory viruses do infect the lower airways,^{13,36} their persistence in respiratory samples in general is of a rather short duration – with the exception of HBoV1.^{14,48,56,74,75} Second, despite high virus-detection rates in both lower and upper airway specimens of asymptomatic children,^{13,15,36} symptom severity is associated with single-virus detections, multiple-virus detections and virus loads, as discussed above.^{14,39–42} Third, PCR findings of HRV and RSV in nasopharyngeal aspirates correlate with the appropriate systemic immune responses in young wheezing children.^{76–78} These data argue against the claim that PCR findings in asymptomatic subjects would be due to residual nucleic acids from preceding respiratory infections.

Table 4

Human rhinovirus detected in upper airway samples of young children with acute wheezing; the risk of recurrent wheezing within 1–2 years and the risk of asthma at age 5–7 years.

1st author (year)	Setting	Age (months)	Index group	Comparator group	Risk of recurrent wheezing within 1–2 years OR or HR (95% CI) adjustment	Risk of asthma at age 5–7 years OR (95% CI) adjustment
Lemanske (2005) ⁸⁸	Outpatients, birth cohort at atopy risk	<12	HRV+ wh+, n=43	HRV+ wh-, n=119	OR 10 (4.1, 26) multiple	-
Lehtinen (2007) ⁸⁹	Inpatients, non-selected population	3–35 (1 st episode)	HRV+wh+ ^a , n=37	RSV+ wh+ ^b , n=43	HR 5.05 (1.00–25.41) Age, atopy, maternal asthma	-
Midulla (2011) ⁹²	Inpatients, non-selected population	<12 (1 st episode)	HRV+ bronchiolitis n=37	acute non-respiratory disease n=39	OR 3.3 (1.0–11.1) multiple	-
Kotaniemi-Syrjänen (2003) ⁸⁷	Inpatients, non-selected population	<24	HRV+ wh+, n=20 ^c	HRV-, wh+, n=43	-	4.1 (1.0 - 17) yes ^d
Kusel (2007) ⁹⁰	Outpatients, birth cohort at atopy risk	<12	HRV+ wh+, n=34	LRI+ wh-, n=193	-	3.2 (1.1 - 9.5) atopy
Jackson (2008) ⁹¹	Outpatients, birth cohort at atopy risk	<12	HRV+ wh+, n=45	HRV+ wh-, n=214	-	2.7 (1.4 - 5.3) yes ^e
Jackson (2008) ⁹¹	Outpatients, birth cohort at atopy risk	12–23	HRV+ wh+, n=37	HRV+ wh-, n=222	-	6.5 (3.1 - 13.7) no

OR, odds ratio; HR, hazard ratio; CI, confidence interval; HRV, human rhinovirus; wh, wheezing; RSV, respiratory syncytial virus; LRI, lower respiratory tract illness.

^a HRV diagnosed alone or together with any other viruses.^b RSV diagnosed alone or together with any other viruses except HRV.^c Single infections.^d Independent of age, sex, and atopic dermatitis.^e Independent of aeroallergen sensitization.

In a symptomatic subject, a positive virus PCR result may identify the causative agent of the current illness (e.g. RSV), a causative agent of an ongoing asymptomatic infection (e.g. HRV), prolonged presence of virus from a past infection (e.g. HBoV), a latent infection or colonization (e.g. adenovirus), or reactivation (e.g. Epstein-Barr virus and human herpesvirus 6). In an asymptomatic subject, a positive virus PCR result, if not due to laboratory contamination, may be due to 1) symptoms and signs that are not recognized, 2) truly asymptomatic acute infection,^{52,79} 3) asymptomatic persistence, or 4) mucosal contamination.

CLINICAL APPLICABILITY OF PCR

All respiratory viruses can be detected by sensitive PCR-based methods. Since acute symptomatic respiratory illness is often accompanied by multiple virus detections in one sample, it may be difficult to prove, against the Koch postulates, that the mere presence of a pathogen would imply that it is the causative agent of the current illness.^{5,14} There is a clear need to move towards quantitative multiplex diagnostics.^{21,80,81} qPCR allows better evaluation of the time course of the infection and of therapy monitoring than end-point PCR. The prevalence of causative viruses exhibits not only seasonal variation, but also considerable year-to-year variation (virus epidemics). The results of a large Swedish study (7853 samples obtained during 36 consecutive months) arrived at a recommendation of using a similar test panel all year round.⁸¹ The routine test panel may, however, need to be redesigned or supplemented by qPCRs, depending on the specific epidemiologic situations to increase sensitivity and enable quantification. Overall, it seems that a high viral load is linked with illness severity and LRTI. Quantitative multiplex PCR may provide an estimate of which viruses are active at the moment and which are emerging or on their retreat. Upper respiratory samples seem to be adequate for clinical routine. Matters that still need elucidation are defining cutoff levels for acute virus infections and standardization of sampling, i.e. how to take comparable respiratory samples over time and interindividually for qPCR.^{63,82} When used together, qPCR and serologic assays provide a more

complete picture of respiratory viral infections, especially HBoV1.⁵⁶

Excluding influenza viruses, there is yet no specific treatment or vaccine available for treating or preventing respiratory virus infections. Viral detection may, however, have practical importance in isolating infected patients in hospitals or in long-term care settings to prevent spread of the contagious disease.⁸³ The diagnosis of a viral infection may also be important in the battle against unnecessary antimicrobial treatments,⁸⁴ although results from a recent report based on 12–36 hour response time in virus detection had no clinical impact in this respect.⁸⁵ Bacterial and viral coinfections may enhance the severity of the illness synergistically by 1) destruction of the respiratory epithelium by the viral infection, which may facilitate bacterial adhesion, 2) virus-induced immunosuppression that may cause bacterial superinfections, and 3) inflammatory response to viral infection, which may upregulate the expression of molecules that bacteria utilize as receptors.⁸⁶ The full extent of the clinical significance of mixed viral–bacterial super- or coinfections remains undecided.

Interestingly, data are accumulating to suggest that the susceptibility to certain viral infections could be a marker of chronic pulmonary inflammatory processes. Paediatric studies have shown a strong link between susceptibility to HRV-induced early wheezing and the development of recurrent wheezing or asthma later in life (Table 4).^{87–92} There is some evidence that young, wheezing children prone to HRV infections will respond to systemic glucocorticosteroid treatment at a high likelihood.^{89,93,94} HRV infections have been linked to an atopic diathesis in young wheezing children,^{11,93,95–98} which partly explains the observation of the effect of systemic glucocorticosteroid treatment.

CONCLUSIONS

All respiratory viruses can be detected with modern, highly sensitive and specific PCR methods, which are likely to detect the causative agent of respiratory infections of patients with or without symptoms. Clinically, multiplex quantitative PCR is currently the most attractive option since it is cost-effective and

the result can be read within hours after sample collection. Because some sensitivity is inevitably lost when multiple primer sequences are included, primers may need redesigning, or multiplex PCR may need to be supplemented by singleplex PCRs, depending on the epidemiological or clinical context. Since multiple viral infections are not uncommon, qPCR may give information on the most active, incipient or waning infections. Combining qPCR and serologic assays provides a more complete picture of respiratory viral infections.

The hurdles of this approach relate to standardization of sampling and to interpretation of multiple virus codetections. Considering that practically all viruses can reach the lower airways and are typically also present in the upper airways, upper airway sampling can be considered adequate for the viral diagnosis of lower airway illnesses. Usually a high virus load and multiple virus detections correlate with lower-airway involvement. Although lower airway sampling, typically bronchoalveolar lavage or induced sputum, increases the sensitivity and specificity of diagnosing lower airway viral infection, these invasive and uncomfortable sampling methods should be reserved for special situations, e.g. for investigating immunocompromised patients in an intensive care unit setting.

Some recent findings suggest that certain viral infections could be markers of pulmonary inflammatory processes. This is especially the case for HRV-induced wheezing which is a high risk factor of childhood asthma. In the future, identification of causative viruses of all hospitalized children with early asthma-like symptoms may be possible and a goal worth pursuing.

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References

- Knipe DM, Howley PM, editors. *Fields Virology*. 5th edition, Philadelphia, PA: Lippincott Williams & Wilkins; 2007.
- Jennings LC, Barns G, Dawson KP. The association of viruses with acute asthma. *N Z Med J* 1987;**100**:488–90.
- Heiskanen-Kosma T, Korppi M, Jokinen C, et al. Etiology of childhood pneumonia: serologic results of a prospective, population-based study. *Pediatr Infect Dis J* 1998;**17**:986–91.
- Jartti L, Langen H, Söderlund-Venermo M, Vuorinen T, Ruuskanen O, Jartti T. New respiratory viruses and the elderly. *Open Respir Med J* 2011;**5**:61–9.
- Jartti T, Waris M, Niesters HG, Allander T, Ruuskanen O. Respiratory viruses and acute asthma in children. *J Allergy Clin Immunol* 2007;**120**:216.
- Cilla G, Oñate E, Perez-Yarza EG, Montes M, Vicente D, Perez-Trallero E. Viruses in community-acquired pneumonia in children aged less than 3 years old: High rate of viral coinfection. *J Med Virol* 2008;**80**:1843–9.
- 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;**28**:311–7.
- Ruuskanen O, Lahti E, Jennings LC, Murdoch DR. Viral pneumonia. *Lancet* 2011;**377**:1264–75.
- Honkinen M, Lahti E, Österback R, Ruuskanen O, Waris M. Viruses and bacteria in sputum samples of children with community-acquired pneumonia. *Clin Microbiol Infect* 2012;**18**:300–7.
- Brand HK, de Groot R, Galama JM, et al. Infection with multiple viruses is not associated with increased disease severity in children with bronchiolitis. *Pediatr Pulmonol* 2012;**47**:393–400.
- Rakes GP, Arruda E, Ingram JM, et al. Rhinovirus and respiratory syncytial virus in wheezing children requiring emergency care. IgE and eosinophil analyses. *Am J Respir Crit Care Med* 1999;**159**:785–90.
- van Gageldonk-Lafeber AB, Heijnen ML, Bartelds AI, Peters MF, van der Plas SM, Wilbrink B. A case-control study of acute respiratory tract infection in general practice patients in The Netherlands. *Clin Infect Dis* 2005;**41**:490–7.
- Malmström K, Pitkäranta A, Carpen O, et al. Human rhinovirus in bronchial epithelium of infants with recurrent respiratory symptoms. *J Allergy Clin Immunol* 2006;**118**:591–6.
- Jartti T, Lee WM, Pappas T, Evans M, Lemanske Jr RF, Gern JE. Serial viral infections in infants with recurrent respiratory illnesses. *Eur Respir J* 2008;**32**:314–20.
- 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;**27**:1103–7.
- Chamberlain JS, Gibbs RA, Ranier JE, Nguyen PN, Caskey CT. Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. *Nucleic Acids Res* 1988;**16**:11141–56.
- Mahony JB, Blackhouse G, Babwah J, et al. Cost analysis of multiplex PCR testing for diagnosing respiratory virus infections. *J Clin Microbiol* 2009;**47**:2812–7.
- Fox JD. Nucleic acid amplification tests for detection of respiratory viruses. *J Clin Virol* 2007;**40**:S15–23.
- Pabbaraju K, Tokaryk KL, Wong S, Fox JD. Comparison of the Luminex xTAG respiratory viral panel with in-house nucleic acid amplification tests for diagnosis of respiratory virus infections. *J Clin Microbiol* 2007;**46**:3056–62.
- Wu W, Tang YW. Emerging molecular assays for detection and characterization of respiratory viruses. *Clin Lab Med* 2009;**29**:673–93.
- Tiveljung-Lindell A, Rotzén-Ostlund M, et al. Development and implementation of a molecular diagnostic platform for daily rapid detection of 15 respiratory viruses. *J Med Virol* 2009;**81**:167–75.
- Earley MC, Vogt Jr RF, Shapiro HM, et al. Report from a workshop on multi-analyte microsphere assays. *Cytometry* 2002;**50**:239–342.
- Kim SR, Ki CS, Lee NY. Rapid detection and identification of 12 respiratory viruses using a dual priming oligonucleotide system-based multiplex PCR assay. *J Virol Methods* 2009;**156**:111–6.
- Roh KH, Kim J, Nam MH, 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.
- Gadsby NJ, Hardie A, Claas EC, Templeton KE. Comparison of the Luminex respiratory virus panel fast assay with in-house real-time PCR for respiratory viral infection diagnosis. *J Clin Microbiol* 2010;**48**:2213–6.
- Arens MQ, Buller RS, Rankin A, et al. Comparison of the Eragen Multi-Code Respiratory Virus Panel with conventional viral testing and real-time multiplex PCR assays for detection of respiratory viruses. *J Clin Microbiol* 2010;**48**:2387–95.
- Rand KH, Rampersaud H, Houck HJ. Comparison of two multiplex methods for detection of respiratory viruses: FilmArray RP and xTAG RVP. *J Clin Microbiol* 2011;**49**:2449–53.
- Raymond F, Carboneau J, Boucher N, et al. Comparison of automated microarray detection with real-time PCR assays for detection of respiratory viruses in specimens obtained from children. *J Clin Microbiol* 2009;**47**:743–50.
- Jansen RR, Schinkel J, Koekkoek S, et al. Development and evaluation of a four-tube real time multiplex PCR assay covering fourteen respiratory viruses, and comparison to its corresponding single target counterparts. *J Clin Virol* 2011;**51**:179–85.
- Pabbaraju K, Wong S, Tokaryk KL, Fonseca K, Drews SJ. Comparison of the Luminex xTAG respiratory viral panel with xTAG respiratory viral panel fast for diagnosis of respiratory virus infections. *J Clin Microbiol* 2011;**49**:1738–44.
- Ali SA, Gern JE, Hartert TV, et al. Real-world comparison of two molecular methods for detection of respiratory viruses. *Virol J* 2011;**8**:332.
- Chan KH, Peiris JS, Lim W, Nicholls JM, Chiu SS. Comparison of nasopharyngeal floxed swabs and aspirates for rapid diagnosis of respiratory viruses in children. *J Clin Virol* 2008;**42**:65–9.
- Spyridaki IS, Christodoulou I, de Beer L, et al. Comparison of four nasal sampling methods for the detection of viral pathogens by RT-PCR-A GA(2)LEN project. *J Virol Methods* 2009;**156**:102–6.
- Munywoki PK, Hamid F, Mutunga M, Welch S, Cane P, Nokes DJ. Improved detection of respiratory viruses in pediatric outpatients with acute respiratory illness by real-time PCR using nasopharyngeal floxed swabs. *J Clin Microbiol* 2011;**49**:3365–7.
- Simons E, Schroth MK, Gern JE. Analysis of tracheal secretions for rhinovirus during natural colds. *Pediatr Allergy Immunol* 2005;**16**:276–8.
- Thavagnanam S, Christie SN, Doherty GM, Coyle PV, Shields MD, Heaney LG. Respiratory viral infection in lower airways of asymptomatic children. *Acta Paediatr* 2010;**99**:394–8.
- Papadopoulos NG, Sanderson G, Hunter J, Johnston SL. Rhinoviruses replicate effectively at lower airway temperatures. *J Med Virol* 1999;**58**:100–4.
- Lahti E, Peltola V, Waris M, et al. Induced sputum in the diagnosis of childhood community-acquired pneumonia. *Thorax* 2009;**64**:252–7.
- Kusel MM, de Klerk NH, Holt PG, Kebabdzic T, Johnston SL, Sly PD. Role of respiratory viruses in acute upper and lower respiratory tract illness in the first year of life: a birth cohort study. *Pediatr Infect Dis J* 2006;**25**:680–6.
- Khetsuriani N, Kazerouni NN, Erdman DD, et al. Prevalence of viral respiratory tract infections in children with asthma. *J Allergy Clin Immunol* 2007;**119**:314–21.
- Bezerra PG, Britto MC, Correia JB, et al. Viral and atypical bacterial detection in acute respiratory infection in children under five years. *PLoS One* 2011;**6**:e18928.
- Utokaparch S, Marchant D, Gosselink JV, et al. The relationship between respiratory viral loads and diagnosis in children presenting to a pediatric hospital emergency department. *Pediatr Infect Dis J* 2011;**30**:e18–23.
- Piralla A, Rovida F, Campanini G, et al. Clinical severity and molecular typing of human rhinovirus C strains during a fall outbreak affecting hospitalized patients. *J Clin Virol* 2009;**45**:311–7.
- Iwane MK, Prill MM, Lu X, et al. Human Rhinovirus Species Associated With Hospitalizations for Acute Respiratory Illness in Young US Children. *J Infect Dis* 2011;**204**:1702–10.
- Fuji N, Suzuki A, Lupisan S, et al. Detection of Human Rhinovirus C Viral Genome in Blood among Children with Severe Respiratory Infections in the Philippines. *PLoS One* 2011;**6**:e27247.

46. Gerna G, Piralla A, Rovida F, et al. Correlation of rhinovirus load in the respiratory tract and clinical symptoms in hospitalized immunocompetent and immunocompromised patients. *J Med Virol* 2009;**81**:1498–507.
47. Bufford JD, Roberg KA, Pappas TE, et al. Quantity of rhinovirus in nasal lavage does not predict illness severity. *J Allergy Clin Immunol* 2007;**119**:S27.
48. Peltola V, Waris M, Österback R, Susi P, Ruuskanen O, Hyypiä T. Rhinovirus transmission within families with children: incidence of symptomatic and asymptomatic infections. *J Infect Dis* 2008;**197**:382–9.
49. Pettigrew MM, Gent JF, Pyles RB, Miller AL, Nokso-Koivisto J, Chonmaitree T. Viral-bacterial interactions and risk of acute otitis media complicating upper respiratory tract infection. *J Clin Microbiol* 2011;**49**:3750–5.
50. Martin ET, Kuypers J, Heugel J, Englund JA. Clinical disease and viral load in children infected with respiratory syncytial virus or human metapneumovirus. *Diagn Microbiol Infect Dis* 2008;**62**:382–8.
51. Jackson ML, France AM, Hancock K, et al. Serologically confirmed household transmission of 2009 pandemic influenza A (H1N1) virus during the first pandemic wave—New York City, April–May 2009. *Clin Infect Dis* 2011;**53**:455–62.
52. Huang Y, Zaas AK, Rao A, et al. Temporal dynamics of host molecular responses differentiate symptomatic and asymptomatic influenza A infection. *PLoS Genet* 2011;**7**:e1002234.
53. Almansa R, Anton A, Ramirez P, et al. Direct association between pharyngeal viral secretion and host cytokine response in severe pandemic influenza. *BMC Infect Dis* 2011;**11**:232.
54. Xu C, Song X, Fu L, et al. Antiviral Potential of Exogenous Human Omega Interferon to Inhibit Pandemic 2009 A (H1N1) Influenza Virus. *Viral Immunol* 2011;**24**:369–74.
55. Dina J, Nguyen E, Gouarin S, et al. Development of duplex real-time PCR for detection of two DNA respiratory viruses. *J Virol Methods* 2009;**162**:119–25.
56. Jartti T, Hedman K, Jartti L, Ruuskanen O, Allander T, Söderlund-Venermo M. Human bocavirus – the first 5 years. *Rev Med* 2012;**22**:46–64.
57. Kalu SU, Loeffelholz M, Beck E, et al. Persistence of adenovirus nucleic acids in nasopharyngeal secretions: a diagnostic conundrum. *Pediatr Infect Dis J* 2010;**29**:746–50.
58. Martin ET, Kuypers J, Wald A, Englund JA. Multiple versus single virus respiratory infections: viral load and clinical disease severity in hospitalized children. *Influenza Other Respi Viruses* 2012;**6**:71–7.
59. Franz A, Adams O, Willems R, et al. Correlation of viral load of respiratory pathogens and co-infections with disease severity in children hospitalized for lower respiratory tract infection. *J Clin Virol* 2010;**48**:239–45.
60. Gerna G, Campanini G, Rognoni V, et al. Correlation of viral load as determined by real-time RT-PCR and clinical characteristics of respiratory syncytial virus lower respiratory tract infections in early infancy. *J Clin Virol* 2008;**41**:45–8.
61. Hyypiä T, Puhakka T, Ruuskanen O, Mäkelä M, Arola A, Arstila P. Molecular diagnosis of human rhinovirus infections- comparison with virus isolation. *J Clin Microbiol* 1998;**36**:2081–3.
62. Puhakka T, Mäkelä MJ, Malmström K, et al. Common cold: Effects of intranasal fluticasone propionate treatment. *J Allergy Clin Immunol* 1998;**101**:726–31.
63. Madeley CR. “Is it the cause?”—Robert Koch and viruses in the 21st century. *J Clin Virol* 2008;**43**:9–12.
64. Esposito S, Bosis S, Niesters HG, et al. Impact of human bocavirus on children and their families. *J Clin Microbiol* 2008;**46**:1337–42.
65. Nascimento-Carvalho CM, Cardoso MRA, Meriluoto M, et al. Human bocavirus infection diagnosed serologically among children admitted to hospital with community-acquired pneumonia in a tropical region. *J Med Virol* 2012;**84**:253–8.
66. Juven T, Mertsola J, Waris M, et al. Etiology of community-acquired pneumonia in 254 hospitalized children. *Pediatr Infect Dis J* 2000;**19**:293–8.
67. Reed C, Kallen AJ, Patton M, et al. Infection with community-onset *Staphylococcus aureus* and influenza virus in hospitalized children. *Pediatr Infect Dis J* 2009;**28**:572–6.
68. Finelli L, Fiore A, Dhara R, et al. Influenza-associated pediatric mortality in the United States: increase of *Staphylococcus aureus* coinfection. *Pediatrics* 2008;**122**:805–11.
69. Juven T, Mertsola J, Waris M, Leinonen M, Ruuskanen O. Clinical response to antibiotic therapy for community-acquired pneumonia. *Eur J Pediatr* 2004;**163**:140–4.
70. Techasaensiri B, Techasaensiri C, Mejias A, McCracken Jr GH, Ramilo O. Viral coinfections in children with invasive pneumococcal disease. *Pediatr Infect Dis J* 2010;**29**:519–23.
71. Talbot TR, Hartert TV, Mitchel E, et al. Asthma as a risk factor for invasive pneumococcal disease. *N Engl J Med* 2005;**352**:2082–90.
72. Juhn YJ, Kita H, Yawn BP, et al. Increased risk of serious pneumococcal disease in patients with asthma. *J Allergy Clin Immunol* 2008;**122**:719–23.
73. Bustin SA, Benes V, Garson. et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin Chem* 2009;**55**:611–22.
74. Jartti T, Lehtinen P, Vuorinen T, Koskenvuo M, Ruuskanen O. Persistence of rhinovirus and enterovirus RNA after acute respiratory illness in children. *J Med Virol* 2004;**72**:695–9.
75. Winther B, Hayden FG, Hendley JO. Picornavirus infections in children diagnosed by RT-PCR during longitudinal surveillance with weekly sampling: Association with symptomatic illness and effect of season. *J Med Virol* 2006;**78**:644–50.
76. Gern JE, Martin MS, Anklam KA, et al. Relationships among specific viral pathogens, virus-induced interleukin-8, and respiratory symptoms in infancy. *Pediatr Allergy Immunol* 2002;**13**:386–93.
77. Gern JE, Brooks GD, Meyer P, et al. Bidirectional interactions between viral respiratory illnesses and cytokine responses in the first year of life. *J Allergy Clin Immunol* 2006;**117**:72–8.
78. Jartti T, Paul-Anttila M, Lehtinen P, Parikka V, Vuorinen T, Simell O, Ruuskanen O. Systemic T-helper and T-regulatory cell type cytokine responses in rhinovirus vs. respiratory syncytial virus induced early wheezing: an observational study. *Respir Res* 2009;**10**:85.
79. Selin LK, Brehm MA, Naumov YN, Cornberg M, Kim SK, Clute SC, Welsh RM. Memory of mice and men: CD8+ T-cell cross-reactivity and heterologous immunity. *Immunol Rev* 2006;**211**:164–81.
80. Lassaunière R, Kresfelder T, Venter M. A novel multiplex real-time RT-PCR assay with FRET hybridization probes for the detection and quantitation of 13 respiratory viruses. *J Virol Methods* 2010;**165**:254–60.
81. Jansen RR, Wieringa J, Koekkoek SM, et al. Frequent detection of respiratory viruses without symptoms: toward defining clinically relevant cutoff values. *J Clin Microbiol* 2011;**49**:2631–6.
82. Brittain-Long R, Andersson LM, Olofsson S, Lindh M, Westin J. Seasonal variations of 15 respiratory agents illustrated by the application of a multiplex polymerase chain reaction assay. *Scand J Infect Dis* 2012;**44**:9–17.
83. Talbot GH. Considerations in undertaking a clinical development program for hospital-acquired bacterial pneumonia and/or ventilator-associated bacterial pneumonia. *Clin Infect Dis* 2010;**51**(Suppl 1):S144–9.
84. Oosterheert JJ, van Loon AM, Schuurman R, 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.
85. Wishaupt JO, Russcher A, Smeets LC, Versteegh FG, Hartwig NG. Clinical Impact of RT-PCR for Pediatric Acute Respiratory Infections: A Controlled Clinical Trial. *Pediatrics* 2011;**128**:e1113–20.
86. Peltola VT, McCullers JA. Respiratory viruses predisposing to bacterial infections: role of neuraminidase. *Pediatr Infect Dis J* 2004;**23**(Suppl):S87–97.
87. Kotaniemi-Syrjänen A, Vainionpää R, Reijonen TM, Waris M, Korhonen K, Korppi M. Rhinovirus-induced wheezing in infancy—the first sign of childhood asthma? *J Allergy Clin Immunol* 2003;**111**:66–71.
88. Lemanske Jr RF, Jackson DJ, Gangnon RE, et al. Rhinovirus illnesses during infancy predict subsequent childhood wheezing. *J Allergy Clin Immunol* 2005;**116**:571–7.
89. Lehtinen P, Ruohola A, Vanto T, Vuorinen T, Ruuskanen O, Jartti T. Prednisolone reduces recurrent wheezing after a first wheezing episode associated with rhinovirus infection or eczema. *J Allergy Clin Immunol* 2007;**119**:570–5.
90. Kusel MM, de Klerk NH, Kebadze T, et al. Early-life respiratory viral infections, atopic sensitization, and risk of subsequent development of persistent asthma. *J Allergy Clin Immunol* 2007;**119**:1105–10.
91. Jackson DJ, Gangnon RE, Evans MD, et al. Wheezing rhinovirus illnesses in early life predict asthma development in high-risk children. *Am J Respir Crit Care Med* 2008;**178**:667–72.
92. Midulla F, Pierangeli A, Cangiano G, et al. Rhinovirus bronchiolitis and recurrent wheezing: one year follow-up. *Eur Respir J* 2012;**39**:396–402.
93. Jartti T, Lehtinen P, Vanto T, et al. Evaluation of the efficacy of prednisolone in early wheezing induced by rhinovirus or respiratory syncytial virus. *Pediatr Infect Dis J* 2006;**25**:482–8.
94. Jartti T, Lehtinen P, Vanto T, et al. Efficacy of prednisolone in children hospitalized for recurrent wheezing. *Pediatr Allergy Immunol* 2007;**18**:326–34.
95. Korppi M, Kotaniemi-Syrjänen A, Waris M, Vainionpää R, Reijonen TM. Rhinovirus-associated wheezing in infancy: comparison with respiratory syncytial virus bronchiolitis. *Pediatr Infect Dis J* 2004;**23**:995–9.
96. Jartti T, Kuusipalo H, Vuorinen T, et al. Allergic sensitization is associated with rhinovirus-, but not other virus-, induced wheezing in children. *Pediatr Allergy Immunol* 2010;**21**:1008–14.
97. Jackson DJ, Evans MD, Gangnon RE, et al. Evidence for a causal relationship between allergic sensitization and rhinovirus wheezing in early life. *Am J Respir Crit Care Med* 2012;**185**:281–5.
98. Miller EK, Williams JV, Gebretsadik T, et al. Host and viral factors associated with severity of human rhinovirus-associated infant respiratory tract illness. *J Allergy Clin Immunol* 2011;**127**:883–91.