

PCR for detection of respiratory viruses: seasonal variations of virus infections

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Real-time PCR and related methods have revolutionized the laboratory diagnosis of viral respiratory infections because of their high detection sensitivity, rapidness and potential for simultaneous detection of 15 or more respiratory agents. Results from studies with this diagnostic modality have significantly expanded our knowledge about the seasonality of viral respiratory diseases, pinpointed the difficulties to make a reliable etiologic diagnosis without the aid of an unbiased multiplex molecular assay for respiratory viruses, and revealed previously unknown details as to possible infections with multiple agents as aggravating factors. The scope of this article is to review and discuss this new knowledge and its implications for diagnostic strategies and other measures essential for the clinical management of respiratory viral infections and for epidemiological surveillance of seasonal respiratory infections.

KEYWORDS: epidemiology • laboratory diagnosis • mixed infections • real-time PCR • seasonal distribution

The advent of real-time PCR and related methods for genome amplification as a sensitive and versatile diagnostic tool have changed our view of the etiology and clinical spectrum of viral respiratory tract infections (RTIs). These tests are relatively cheap, rapid, sensitive and suitable for the analysis of a respiratory specimen for 15 or more agents assayed simultaneously. This has prompted clinicians to submit a larger number of specimens for virological examination than ever expected. The most important lessons learned from the last few years of organized molecular diagnosis of respiratory viruses are that it is more difficult than anticipated, even for an experienced clinician to make an etiologic diagnosis based on clinical data alone, and that positive specimens may often contain two or more respiratory viruses. This indicates that several viruses may replicate in the respiratory tract at the same time, with the potential to contribute to a variety of symptoms. The scope of this article is to present the new molecular techniques for detection of respiratory viruses, and to review new insights into the epidemiology and seasonal appearance of RTIs generated by the new diagnostic tools.

Procedures for molecular diagnosis of respiratory viruses

The methods used for laboratory diagnosis of respiratory viruses today are almost exclusively

based on nucleic acid-amplifying methods, where real-time PCR or multiplex PCR techniques with liquid array (bead-based) detection systems play a dominating role. Owing to the high analytical sensitivity, rapidity, multiplexing capacity and increasing affordability of the new molecular methods, older techniques using virus isolation in cell culture, antigen detection via enzyme immunoassays or immunofluorescence microscopy, and conventional qualitative PCR techniques have in essence become obsolete for everyday clinical practice [1–3]. However, direct immunofluorescence of specimen smears (direct fluorescent antibody [DFA]) is still used in some laboratories owing to its specificity, rapidity and cost-effectiveness, but its sensitivity is consistently lower than that offered by most molecular methods [4–10]. Virus isolation in cell culture and related techniques may remain important in special situations, for example in order to identify unknown infectious agents, where identification of the SARS corona virus is an important example. Moreover, a clinical isolate provides infectious material that could be used for biological analysis, including phenotypic determination of, for example, resistance to antiviral treatment.

The real-time PCR procedures used in the diagnostic laboratory use reporter systems based either on hydrolysis probes [11,12], molecular beacons [13] or fluorescence resonance

energy transfer probes [14]. Each of these probe systems is associated with its own advantages and disadvantages [15,16]. In-house systems based on real-time PCR utilize a combination of three to six multiplex PCR reactions, with the capacity to assay for 15 or more different respiratory agents in a sample. A reverse transcriptase step prior to PCR is required for the detection of genomes of RNA viruses. The multiplex systems linked with liquid array (i.e., bead-based) can theoretically resolve up to 100 independent channels, whose capacity is only partly utilized in the current commercial systems, managing analysis of more than 20 different agents in the same sample. The performance of three such commercial systems were recently compared [17]. A summary of different nucleic acid amplification systems is presented in TABLE 1, but readers are referred to recent excellent reviews of the subject for a more comprehensive comparison of available in-house and commercial systems for molecular diagnosis of respiratory viruses [18–22].

Besides the PCR-based methods, a number of isothermal nucleic acid amplification techniques have been implemented in laboratory diagnosis of respiratory viruses. Nucleic acid sequence-based amplification has been utilized in multiplex real-time methods in conjunction with specific reporter probes for parallel detection of nine respiratory viruses [23,24]. Loop-mediated

isothermal amplification is another isothermal amplification method that has been considered as a tool in the laboratory diagnosis of viruses [20].

The agents targeted in most commercial and in-house systems include influenza virus A and B (IFA, IFB), respiratory syncytial virus (RSV), human metapneumovirus (HMPV), parainfluenza-virus 1–3 (PIV1–3), adenoviruses (AdV), human rhinoviruses (HRV), human enteroviruses (HEV) and human coronaviruses (HCoV). Some systems also include assays for human bocavirus (HBoV) [25] and human parechovirus (HPeV) [26]. AdV, HEV and HRV are usually not further typed [3,27–29]. Most systems include separate assays for two or more of the human coronaviruses NL63, HKU1, 229E and OC43. Bacterial targets (mostly *Chlamydia pneumoniae* and *Mycoplasma pneumoniae*) are included in some systems [11].

Practical aspects on molecular diagnosis of infections with respiratory viruses

Disease episodes & virus shedding

Clinical specimens sampled with correct technique early after the appearance of symptoms are a prerequisite for reliable laboratory diagnosis of RTI [18]. This is also relevant for disease episodes with a longer duration of symptoms. Thus, the detection frequency

Table 1. PCR methods used in laboratory diagnosis of respiratory viruses.

Method	Description	Comments
Conventional PCR	Usually evaluated by colorometry or agarose gel electrophoresis	No quantitation High risk of cross-contamination, especially when using nested format Obsolete
Real-time PCR	PCR products evaluated in real time during each PCR cycle Evaluation either by colorometry or by specific, light-emitting probes (e.g., hydrolysis or FRET probes)	Suitable for limited multiplexing Permits quantitation or semiquantitation of virus Highly specific provided that specific probes are used Very low risk for cross-contamination Excellent for in-house tests, resulting in an affordable analysis for the clinic
Multiplex PCR linked with liquid array (Luminex®; Luminex Corporation); commercial systems: X-TAG® RVP (Abbott) ResPlex® (Qiagen) MultiCode®-PLx (EraGen Biosciences)	Traditional PCR designed to affix amplicons to specific beads in a highly multiplexed format	Excellent multiplexing Several commercial tests available No quantification option Exogenous handling of amplified materials results in risks for cross-contamination Need for commercial systems not cost-compatible
NASBA; LAMP	Isothermal cyclic amplification involving DNA (LAMP) or RNA/DNA (NASBA) intermediates	No need for thermal cyler No reverse transcriptase step for RNA viruses† High sensitivity Suitable for in-house tests, resulting in an affordable analysis for the clinic Quantitation cumbersome
Rapid diagnostic PCR instruments: Xpert® (Cepheid) FilmArray® (Idaho) Jaguar® (BD diagnostics) Infiniti® (AutoGenomics)	Integrated extraction and PCR run in preloaded reaction cassettes, requiring a minimum of hands-on time	No specimen batching needed Suitable for point-of-care testing Locked to commercial assays linked to the instrument Relatively expensive

†Nucleic acid sequence-based amplification only.

For references, see section 'Procedures for molecular diagnosis of respiratory viruses' in text.

FRET: Fluorescence resonance energy transfer; LAMP: Loop-mediated isothermal amplification; NASBA: Nucleic acid sequence-based amplification.

for enveloped respiratory viruses decreased from more than 51 to 30% for specimens taken from patients within 0–6 versus 7–14 days of symptom duration, respectively [30]. A similar decline in the detection frequency over time has also been observed for RSV [31,32]. In the study by Brittain-Long *et al.*, a linear increase in threshold cycle (Ct) value dependent on the duration of symptoms was observed for the majority of enveloped respiratory viruses [30]. This suggests a gradual decrease in the amount of virus that was shed even if clinical symptoms remain. In the same study, there was a moderate increase in the Ct value for HRV in the cases that were still PCR-positive after 10 days, indicating that replication during HRV infection declines slowly. This is concordant with the finding that children may shed HRV and HEV from the respiratory mucus for several weeks [33]. Some respiratory viruses, such as bocaviruses, may be shed for several months [34].

Significance of a positive test result

It is justified to conclude that molecular diagnostic procedures for viral respiratory diseases have unparalleled detection sensitivities in comparison with older techniques [18,19]. An obvious risk with a sensitive assay is that a positive result may reflect a clinically irrelevant carriership rather than a symptomatic infection. This risk appears to be limited among adult individuals for most respiratory viruses: all but two (HRV in both cases) specimens from 100 healthy Swedish control subjects were negative for respiratory viruses [30], and in a similar British study, 2 and 6% of the healthy controls were positive for HRV or IFA, respectively [35]. The observed higher rate of detection of HRV reported for healthy children may represent either truly asymptomatic carriership or, more likely, prolonged postsymptomatic shedding of picornavirus [36–39]. Thus, a nonsymptomatic status is usually defined by the absence of symptoms up to 1 week before and 1 week after the time for sampling [38]. However, HRV may remain detectable by PCR methods for 2 weeks or more after the onset of symptoms [30]. Immunocompromised individuals may shed detectable amounts of respiratory viruses, for example RSV, in the absence of respiratory symptoms [40,41]. Moreover, it should be kept in mind that some respiratory viruses, above all HBoV in infected children, may be secreted for several months [34,42,43], and that therefore additional diagnostic measures must be included in these cases to demonstrate an etiological relationship to respiratory symptoms (see section ‘Newly discovered respiratory viruses’). In conclusion, quantitative methods, such as real-time PCR, offer additional diagnostic value over nonquantitative methods, because the quantity of the agent detected may aid in distinguishing a true etiological agent from an innocent bystander [27,44].

A broad diagnostic panel, preferably consisting of 15 or more agents, also offers an additional diagnostic value: first, a negative result is more valid if many agents have been targeted. Second, the quantitative component of the test improves the interpretation of a positive result, not least if several agents are detected and the Ct values of these agents can then be compared [30]. As described in more detail later (see section ‘Seasonal distribution of respiratory viruses’), it is clear that the degree of association with respiratory

symptoms for individual viruses is variable, where for example, IFA and RSV are more firmly associated with clinical symptoms than many other viruses, suggesting that even low IFA and RSV RNA levels may be of clinical relevance. Sometimes, antigen detection methods, above all direct immunofluorescence techniques (DFA), are considered to be superior to molecular methods because the latter are claimed to be too sensitive [5]. According to this view, highly sensitive molecular methods in general may detect not only higher quantities of viruses that represent a true etiological agent, but also lower, irrelevant, quantities of passenger viruses. However, it appears unlikely that the detection sensitivity of DFA by definition would be able to exactly pinpoint the clinically significant level for each of the 15 or more respiratory agents in all types of patients. By contrast, by virtue of its high detection sensitivity, a quantitative molecular method may be standardized, based on clinical studies, to define individually clinically relevant cutoff levels for adequate laboratory diagnosis, adapted to cope with most of the many combinations of individual agents and different clinical situations induced. However, more clinical studies are required in order to explore the full diagnostic capacity of multiplex quantitative assays. In conclusion, quantitative molecular methods have a great potential to identify a causative viral agent in most cases, provided that sampling from nasopharynx and/or oropharynx is performed early in the course of the disease, when the virus concentration in these compartments is high [11].

Arguments for unbiased approach with broad diagnostic panels

Before molecular tests for respiratory viruses became available, most specimens sent for virological analysis were assayed for, at best, up to five different agents, often with immunofluorescence or other methods with low detection sensitivities [45]. The advent of multiplex molecular tests, expanding the diagnostic arsenal of assays with the capacity to detect 15–20 different viral agents simultaneously with excellent detection sensitivities, has not only significantly increased the rate of virus detection, but also improved our understanding of the etiology of RTIs. Two important lessons have been learned. First, viruses previously believed to cause exclusively mild upper RTIs, including HRV and HCoV, may be associated with severe disease, affecting the lower respiratory tract [46–48]. Second, it is more challenging than previously anticipated to make an etiologic diagnosis judging from the clinical presentation [49–51]. This knowledge combined with the continuously decreasing cost per analysis of molecular tests strongly supports increased testing of specimens from patients with respiratory symptoms against a large panel of respiratory viral agents and, in addition, bacteria giving rise to atypical respiratory symptoms, such as *C. pneumoniae* and *M. pneumoniae*.

Cost aspects of laboratory diagnosis of respiratory viruses

Direct fluorescent antibody is generally considered to be an inexpensive technique compared with other molecular tests available [5], but the costs of molecular tests are steadily decreasing, owing to multiplexing, decreased reaction volumes and an

increasing degree of automation. Thus, a multiplex real-time PCR service, covering 15 respiratory agents [11], with every day service can be offered to the clinician at a cost of €35 per specimen. Furthermore, if hospitalization and the cost of other healthcare-related procedures is considered, a molecular method is the most cost-efficient modality for laboratory diagnosis of respiratory infections, even if a commercial assay was used [52]. Recent data suggest that access to multiplex molecular tests with the capacity to identify 15 respiratory agents may substantially reduce the use of antibiotics [53], but there are also data suggesting that such antibiotic cost reductions based on intensified molecular diagnostics are not easily achieved [54].

Seasonal distribution of respiratory viruses

The patterns of seasonal variations of respiratory viruses, discussed for individual virus species later, are complex, with similarities as well as differences between the various virus families harboring respiratory agents. A brief summary of the data presented for individual viruses in this article are presented graphically in FIGURE 1.

Respiratory syncytial virus & human metapneumovirus

Respiratory syncytial virus and HMPV both belong to the *Pneumovirinae*, subfamily under the virus family *Paramyxoviridae*, comprising large enveloped negative strand RNA viruses. RSV is regarded not only as the most common cause of bronchiolitis in children, but also as a frequent cause of other types of lower RTIs [55,56]. RSV exists as two subgroups A and B; RSV subgroup A was previously believed to give rise to more severe symptoms [57]. However, this has not been confirmed in more recent studies [58,59]. The relative prevalence of RSV subgroup A and B changes from year to year and may also vary in a geographical manner [58–60]. HMPV infects all ages and greatly resembles the clinical presentation of RSV, with symptoms ranging from milder upper RTI symptoms to bronchiolitis and pneumonia [45,56,61].

Some studies find that HMPV infections are much less frequent than RSV infections [62,63], whereas others report equal prevalence for HMPV and RSV infections [64].

The seasonal distribution of RSV and HMPV infections overlap as a broad peak during the winter/early spring period, usually from December through March in temperate climates [62,64], although a more extended season has been suggested for HMPV [65]. Long-term studies over several consecutive years suggest large variations in the detection rate of HMPV [46,63], compared with relative large yearly winter outbreaks of RSV during the last few seasons at least in Northern Europe [62,63]. Previous results indicating that more severe RSV epidemic seasons occur in a biannual mode [66,67] have not yet been fully confirmed with molecular methods [11,28,68].

Parainfluenzavirus 1–3

Similar to RSV and HMPV, PIVs also belong to the paramyxovirus family and have a similar morphology and genome organization. PIVs comprise four different serotypes/genotypes with different clinical spectra [19,69]. Thus, PIV1 and PIV2 are the major causes of croup in young children [70], but may also cause upper RTI in various age groups [46,69]. PIV3, on the other hand, is associated with more severe lower RTI in children, whereas PIV4 (divided into PIV4a and PIV4b subtypes) infections are less common, with a largely unknown epidemiology [19]. Taxonomically PIVs form two genera within subfamily *Paramyxovirinae*, in the *Paramyxoviridae* family – that is *Rubulavirus* (PIV2 and PIV4a/PIV4b) and *Respirovirus* (PIV1 and PIV3) [69].

Fox demonstrated that the PIV seasons (including PIV1, 2 and 3) in Canada peaked in April in 2004 and 2005, and in January in 2006 [68]. In a Swedish clinical series looking at November 2006 to October 2009, we found that the peak season for PIV was from April to June [62]. Although none of these studies reported data for the seasonal distribution of individual PIV types, they suggest that the PIV season in temperate regions take place later in the spring than is described above for RSV and HMPV.

Influenza viruses

Human influenza viruses A, B, and C (IFA, B and C) are enveloped negative strand viruses with segmented genomes belonging to the family *Orthomyxoviridae*. IFA is divided into discrete subtypes, designated according to the identity of the hemagglutinin (H) and neuraminidase (N) envelope glycoprotein genes. Seasonal human influenza viruses during the last 100 years include three subtypes: H1N1, H2N2 and H3N2. A new H1N1 variant transferred from swine to humans was responsible for a pandemic outbreak during 2009 [71]. Cross-species transfers of IFA (above all H5N1) from birds to humans have resulted in severe outbreaks, preferentially

Virus	Month											
	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
RSV		Blue	Blue									
HMPV		Blue	Blue									
HCoV		Blue	Blue									
Picornia		Blue	Blue						Blue			
AdV						Blue	Blue	Blue				
IFA		Blue	Blue									Blue
		Red	Red	Red	Red	Red	Red	Red	Red	Red	Red	Red
					Green	Green	Green	Green	Green			

Figure 1. Schematic overview of respiratory virus seasonality. Intensity of color represents number of positive detections. Blue: temperate regions, Northern Hemisphere. Green: temperate regions, Southern Hemisphere. Red: tropical regions. AdV: Adenoviruses; HCoV: Human coronaviruses; HMPV: Human metapneumovirus; IFA: Influenza virus A; RSV: Respiratory syncytial virus. See section ‘Seasonal distribution of respiratory viruses’ for references.

in Asia [72], but the ability of *bona fide* avian viruses (H4–H16) to spread between humans appears to be limited, owing to different tissue tropisms of human and avian IFA viruses [73,74]. The classical respiratory symptoms of influenza are cough, sore throat, rhinorrhea and nasal congestion, combined with systemic symptoms, such as fever, headache, myalgia and malaise [45,46]. In contrast to IFA, IFB only gives rise to local outbreaks. IFC is rare and associated with mild disease, and is not included in the majority of molecular panels. Most molecular tests do not subtype IFA in the first run; only specimens positive for IFA are subjected to subtyping with respect to the H gene identity.

The seasonal IFA virus in the Northern hemisphere temperate zones usually peaks between December and February. The seasonal IFA epidemics vary greatly in intensity. Thus, the IFA season of 2008–2009 in Sweden was much more intense than the two previous seasons in terms of positive specimens submitted to the laboratory [62], whereas a large German study identified 2002–2003 and 2004–2005 as intense IFA seasons compared with previous seasons from 1997 [28]. In the temperate regions of the Southern Hemisphere (35°S) the seasonal IFA virus usually peaks June–July and is rarely found November–March [75]. In the subtropical parts of Asia, IFA may occur throughout the entire year with sporadic examples of larger outbreaks of seasonal IFA, such as in August–September 2007 and 2008 [76]. The corresponding outbreaks of IFA in an Indian population reached their maxima in June–July in 2007 and 2008, respectively, correlating positively with the rain season [77]. These studies indicate that climate-related factors have a great impact on the timing of the yearly IFA season.

In 2009, the seasonal influenza incidence was influenced by the pandemic H1N1 influenza, resulting in replacement of seasonal H1N1 virus and to a lesser extent also of H3N2 virus in Australia [78]. During the first part of the pandemic, a Swedish study demonstrated that only a few of the symptomatic travelers returning from locations with ongoing pandemic H1N1 were positive for IFA, whereas 60% of the travelers were positive for other respiratory viruses, mostly HRV because a heavy HRV outbreak with IFA-like symptoms happened to coincide with the early phases of the H1N1 epidemic in Sweden [79]. This finding strongly supports the notion that specimens should be tested for multiple respiratory agents even when epidemiological surveillance presents evidence for an upcoming pandemic situation.

A general picture from three independent studies, representing samples from temperate regions, is that the season for IFB coincides with that discussed for PIV above – that is with peak levels in March and April – although not every year presents IFB peaks [28,63,68].

Coronaviruses

Coronaviruses are enveloped RNA viruses with a large genome. Besides the SARS coronaviruses, which cannot be regarded as a seasonal virus, there are four HCoV, originally divided into two serogroups (the third serogroup does not contain any human viruses), that contribute to seasonal outbreaks – that is HCoV-229E and NL63 of Group I and HCoV-OC43 and -HKU-1 of Group II [46].

These serogroups have been confirmed phylogenetically, and recently the coronavirus groups were proposed by the Coronavirus Study Group of the International Committee for Taxonomy of Viruses to be classified as three genera *Alphacoronavirus*, *Betacoronavirus* and *Gammacoronavirus*, respectively [80]. All of these HCoVs cause mainly upper RTI symptoms, but have the capacity to cause more serious lower RTI manifestations in children, as well as adults [81,82]. HCoV-NL63 seems to differ from the other types regarding an ability to induce croup in children [83], and in two publications the majority of HCoV-229E-positive samples were collected from immunocompromised patients [84,85].

There are only a few studies in which the prevalence of HCoV infections has been studied continuously for several years with molecular methods. In a British study of 2003–2006, a typical seasonality of HCoV was found with a peak in February–March, although sporadic cases of HCoV-229E occurred in the early autumn one year [84]. A similar distribution was found in a Swedish study with specimens collected from 2006 to 2009, although the maximum HCoV-OC43 prevalence occurred somewhat earlier than for HCoV-NL63 [63]. However, the peaks of two major HCoV outbreaks in the Beijing (China) area in 2005 and 2007 occurred in September–October and May–July, respectively [86]. Data from juvenile, as well as adult, patients in Hong Kong over 2 years revealed that HCoV-OC43 peaked in November–December, whereas HCoV-NL63 was frequent in July–October [87,88]. This is in line with observations for other respiratory viruses (see previously) that the seasonality is strongly dependent on the geographical location. The four HCoVs do not seem to appear simultaneously and evidence is accumulating that at least a few HCoV types present in a biannual mode [86,88].

Rhinoviruses, enteroviruses & parechoviruses

The virus family *Picornaviridae*, including small nonenveloped, icosahedral, positive-strand RNA viruses, to which the former genera human rhino-, entero- and parecho-viruses (HRV, HEV, and HPeV, respectively) belonged, has recently been reclassified by the International Committee on Taxonomy of Viruses. According to the current taxonomy, HRV no longer forms a genus of its own, but belongs to the genus *Enterovirus* as three species HRV-A, HRV-B and HRV-C, each one containing numerous variants, referred to as ‘serotypes’, ‘types’ and so on [89–92]. The original members of the *Enterovirus* genus now form four species: HEV-A–D belonging to the enlarged *Enterovirus* genus [91]. HRV are true respiratory viruses and many studies suggest that this agent is the most common cause of common colds, but HRV may also give rise to lower RTIs, as well as exacerbations of asthma and chronic obstructive pulmonary disease [44–46,68,90,93–97]. HRV is the most prevalent respiratory virus in children under the age of 5 years and the leading cause of hospitalization of children under the age of 2 years with wheezing [93–95,98,99]. Recent clinical studies suggest that HRV-C is found considerably more frequently than anticipated in hospitalized children [100–102]. Most molecular methods applied do not permit HRV typing. Design of a pan HRV-A, -B and -C real-time PCR method is complicated by sequence variations among the various HRV types that such a test must cover [11,18,28,46].

In contrast to many other respiratory viruses, HRVs are frequently found in clinical specimens all year around in temperate regions with occasional peaks [28,62]. There seem to be a higher frequency of asymptomatic carriers, positive for HRV than for other respiratory viruses, possibly owing to a longer period of viral shedding of HRV compared with many other respiratory viruses [30,97].

Although HEV-A, -B, -C and -D are considered to be enteric viruses, they may cause upper RTI infections, not least in children [103,104]. The respiratory HEV incidence pattern is similar to that of HRV [28].

The prototype strains of HPeV-1 and -2 were previously considered to be enteroviruses (Echovirus-22 and -23) but were renamed in 1999 owing to differences in genomic structure and other properties [105], and *parechovirus* maintains its status as a genus within *Picornaviridae* [91]. HPeVs are genetically and antigenically heterogeneous and circulate in the human population worldwide, preferentially infecting infants and young children [106]. A broadly specific real-time PCR detecting all known HPeV types is now available [26]. Early retrospective studies suggested that HPeV, besides being the cause of gastrointestinal symptoms, may also be the prominent cause of respiratory symptoms [107]. Some recent studies involving molecular methods support a connection between HPeV and respiratory disease [108,109], but this conclusion is somewhat obscured by the lack of data regarding possible concomitant infections with other agents. In one larger British study where respiratory specimens were analyzed, it was found that HPeV of types 1 and 6 showed a seasonal pattern, peaking in July and August, infecting mainly children below 5 years, and this was most likely not associated with the respiratory disease that brought the children to a physician [110]. It is obvious that further studies are needed to evaluate the potential relationship between HPeV infections and respiratory disease.

Adenoviruses

Adenoviruses are large double-stranded, nonenveloped DNA viruses belonging to the *Adenoviridae* family, where all the human adenoviruses belong to the genus *Mastadenovirus*. Of more than 50 adenovirus serotypes, belonging to the *Mastadenovirus* species A–F, at least nine different types can infect the respiratory tract, primarily in children [46]. The symptoms are usually mild, but AdV may also cause more severe manifestations, including croup, bronchiolitis and pneumonia [89]. Most systems for molecular diagnosis of AdV are type-common and do not distinguish between different serotypes [28,62,68,111].

The incidence of AdV infections is high throughout the year in temperate regions, with a slight decrease during the summer months [62].

Newly discovered respiratory viruses

During the last decade, several novel respiratory viruses have been detected using conventional cell culture coupled with other conventional methods, such as immunofluorescence, as well as molecular techniques. Recent successful molecular strategies include elaborate biochemical approaches to concentrate virus particles in respiratory specimens followed by analysis with advanced

algorithms for recognizing viral sequences after degenerate PCR of the enriched virus preparations [112,113]. The clinical significance of these agents is currently under evaluation by molecular diagnostic methods applied in large clinical series. The significance of a few of these new agents is discussed here.

Human bocavirus

Human bocavirus belongs to the family *parvoviridae* and is a small nonenveloped, icosahedral virus with a single-stranded DNA [114]. HBoV was discovered by Allander and coworkers using a virus discovery strategy, based on DNA depletion, random PCR amplification, large-scale sequencing and bioinformatics, on pooled respiratory extracts [115]. A number of clinical studies have reported a statistical association between HBoV infection and acute respiratory symptoms (cough, acute wheezing, pneumonia and so on) in a manner that may suggest a causal role [116–118], but evidence has also accumulated that HBoV infection may take place without any symptoms in many cases [114]. A sensitive real-time quantitative PCR method was recently developed, not only identifying HBoV, but also three other newly discovered enteric bocaviruses, designated HBoV2, HBoV3 and HBoV4 [25].

Recent data suggest that only primary infections, but not respiratory tract re-infections, with HBoV are associated with viremia and respiratory tract symptoms, such as wheezing in children. Therefore, the detection of HBoV-DNA in nasopharynx by PCR is not adequate for laboratory diagnosis of HBoV infections, but it has been recommended to analyze HBoV DNA in plasma or IgM antibodies to demonstrate HBoV as an etiological agent for respiratory tract infections [43,119,120]. Viremia seems to be an infrequent phenomenon for most other respiratory viruses described previously [121], only sporadically reported in patients with severe IFA or infants infected with RSV [122,123], but even for these two latter situations PCR testing of nasopharynx specimens appears to be adequate for laboratory diagnosis. Thus, HBoV therefore differs from most other respiratory viruses regarding the additional strict requirement of serology and/or viremia assays for etiological confirmation.

The seasonality of HBoV infections are under debate where some studies suggest a high incidence during late spring and early summer [124,125], whereas others fail to reveal any seasonality, although the overall number of cases may vary between years [126].

KI & WU viruses

Two new human polyoma viruses, KI and WU, present in human respiratory specimens were identified in 2007 [127,128]. A number of studies from different regions suggest that KI or WU polyoma viruses are detectable in 1–5% of the specimens submitted for laboratory diagnosis of respiratory disease, but the clinical significance of these findings remains to be determined [129–131]. There seem to be a seasonal variation of WU, but not KI, polyoma virus, with a peak from late winter to early summer in Australia [129].

Clinical significance of multiple agents

One advantage of the molecular methods as tools for laboratory diagnosis of respiratory infections is their ability to detect multiple agents in a single specimen [51]. This is especially relevant for

real-time PCR techniques whose combination of a high specificity and wide dynamic range allows the identification of even minor populations of coinfecting viruses. Multiple respiratory viruses have been detected in approximately 10% of the respiratory specimens [46], with higher rates recorded in young children and immunosuppressed patients. The significance of this finding is uncertain, but infection with multiple viruses has been associated with more severe clinical symptoms [27,110,114,132]. For example, the course of RSV bronchiolitis in infants may be aggravated by coinfection with HRV or HMPV [132]. A similar relationship has been suggested for HBoV, as well as HPeV and classical enteroviruses [110,114]. However, during the winter season when several respiratory viruses may cause parallel outbreaks, children attending daycare centers can harbor up to four different respiratory viruses simultaneously at different concentrations. Further studies are needed to reveal the clinical implications of such multiple infections, and it is likely that quantification of the relative concentrations of the agents detected by real-time PCR may prove to be useful in the assessment of their clinical significance [11,27].

Expert commentary & five-year view

It is evident that molecular methods have revolutionized diagnostics of viral respiratory diseases, not only because of the new unparalleled detection sensitivities achieved, but also because of the option of simultaneous assay for a great number of agents, at a reasonable cost. This has resulted in a new and more detailed picture of the seasonal covariations of at least a dozen of the most frequent viral respiratory agents and, not least, an improved understanding of the clinical significance of infections with multiple agents. One of the most important lessons that can be learned from the past 10 years of multiplex molecular diagnosis of respiratory viruses is that all specimens submitted for laboratory diagnosis of respiratory agents should be assayed for the full panel of respiratory viruses. First, the data accumulated clearly demonstrate that it is extremely difficult to make an etiological diagnosis based on symptoms and other clinical data. Second, viruses previously considered to induce only mild upper RTI symptoms are not seldom associated with more serious lower RTI symptoms. Third, it may be important to identify infections with two or more respiratory viruses because the clinical effects generated by one virus may be amplified by coinfection with another virus.

In parallel with the development of multiplex molecular assays with the capacity to concomitantly analyze a growing number of different respiratory agents, there is a development of 'in-depth' multiplex methods with the capacity to, for example, subtype the influenza virus and even identify treatment-resistant variants in specimens taken directly from the patient [133]. It is likely that this line of development will involve respiratory viruses other than IFA, should new types of therapy become available.

For these reasons it could be anticipated that the general demand for laboratory diagnosis of viral RTIs based on multiplex molecular methods will increase during the next few years, and that this development will be promoted by reduced costs for this type of laboratory service. We will probably also see more rapid bedside diagnosis of viral RTIs based on multiplex molecular

methods, that are able to fill the gap between the laboratory-based PCR methods that require several hours until the clinician gets a report and the current rapid bedside antigen detection tests, such as DFA, with lower sensitivity and specificity. There are already second-generation types of multiplex equipment available that integrate nucleic acid extraction/purification with PCR for a few respiratory agents. These assays provide results within 1 h, but improvements regarding performance, range of agents addressed and capacity to accommodate a higher number of specimens can be anticipated.

Today most clinical laboratories use in-house tests for molecular diagnosis of viral RTIs. Although the supply of different types of commercial molecular tests for viral RTIs are expected to increase, it is reasonable to assume that the in-house tests will be competitive, at least for the larger hospital laboratories, not only for economic reasons. One advantage of in-house tests is that whenever a new infectious agent is detected, it is easy to rapidly develop an in-house molecular test as soon as adequate sequence data are available in GenBank, whereas the development of commercial tests, robust enough to also be used in laboratories lacking necessary experience in diagnostic molecular biology, for obvious reasons is more time consuming. One previous drawback associated with in-house assays – lack of standardization – is now being overcome owing to rapid online publishing of new methods and international standardization networks between university hospital laboratories [134]. Many diagnostic virological laboratories have included assays for bacteria causing RTIs with virus-like symptoms, and it is likely that this development line will continue.

The advent of improved methods for nucleic acid sequencing ('Next Generation Sequencing' [NGS]; reviewed in [135]) will probably be able to supplement the PCR-based laboratory diagnosis of viral RTIs both because of the high capacity of the new technology and the steadily decreasing price per sequenced base pair. Recent NGS platforms developed for smaller laboratories will probably soon be able to sequence and report up to 10 gigabases within 1–2 days, at a cost today of less than €1000. Sequence analysis will probably only be applied to a minority of the specimens, but will be able to tentatively define the 'population respiratory virome' circulating during a RTI season. Obvious advantages associated with NGS sequencing of respiratory specimens are the capacity to discover new respiratory viral agents and the occurrence of chemotherapy-resistant viral variants.

Molecular methods have revealed that multiple respiratory virus infections are more frequent than previously expected. More studies are needed in order to reveal to what extent coinfection or superinfection with additional respiratory viruses contribute to the clinical picture and to explore whether the quantitative option of real-time PCR can be used to determine the clinical relevance of such multiple infections. Further study should also be conducted to evaluate to what extent a primary infection with one respiratory virus increases (or decreases) the susceptibility to infection by other respiratory agents [136].

For the surveillance of seasonal respiratory virus infections it is of importance to identify the geographical locations of the long-term sources from which the respiratory virus outbreaks in

temperate regions are recruited. Genomic and epidemiological dynamics data for IFA suggest a sink-source model of viral ecology, postulating that new viral lineages are seeded from a tropically situated influenza reservoir to the population (sink) in the temperate regions [137]. One important future use of the new molecular methods may be as epidemiological tools to reveal the nature and location of persistent sources of other seasonal respiratory viruses and the routes by which they reach their temperate sink populations. At present, we do not know whether the specific climatology or the high population density of the tropical regions constitute the basis for the IFA source function of this region. Neither do we know whether different seasonal viruses of the temperate regions arise from the same source regions, nor if, for example, RSV and PIV outbreaks originate from different, unique geographical source locations. In addition, we currently know very little about how seasonal outbreaks are influenced by large-scale factors, such as ecological conditions, possible interference between different

respiratory viruses in the human population and, not least, natural disasters. There are reasons to believe that the global use of molecular PCR methods for laboratory diagnosis of viral RTIs in parallel with NGS methods for sequencing of selected clinical specimens will be helpful in a 5-year perspective to define not only important respiratory virus sources with respect to the geographical location and the human populations involved, but also the epidemiological mechanisms by which respiratory outbreaks are generated and terminated in the population of temperate regions.

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Key issues

- New molecular methods for genome amplification, including real-time PCR, have resulted in an improved understanding of the etiology and clinical spectrum of viral respiratory tract infections.
- The most important advantages of these tests are that they are rapid, sensitive, cheap and that they offer multiplexing with an capacity to assay for more than ten different respiratory agents simultaneously.
- Most large clinical laboratories use in-house tests, but there are also high-quality commercial tests available.
- Almost 10 years of experience of the new molecular methods has demonstrated that viruses previously believed to cause exclusively mild upper respiratory tract infections may be associated with severe disease, and that it is more difficult than previously realized to make an etiologic diagnosis judging from the clinical presentation.
- The data that have been accumulated with the new molecular methods strongly support increased testing of specimens from patients with respiratory tests with a large panel of viral agents.
- Although there are differences in the fine structure of the seasonal distribution between different viruses, it is a general impression that most enveloped respiratory viruses are detected most frequently from January to March in the temperate climate region of the Northern hemisphere, whereas nonenveloped viruses, such as rhinoviruses and adenoviruses, are distributed more evenly over the year and enteroviruses cause outbreaks in the autumn.
- Respiratory viruses are easily detected around the year in the tropical and subtropical regions, and it is possible that these regions serve as reservoirs for the respiratory virus variants that cause the seasonal outbreaks in the temperate regions.

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