Elucidation and Clinical Role of Emerging Viral Respiratory Tract Infections in Children

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Abstract

Acute respiratory viral infections (ARVI's) are the most common infectious disease in humans. With the appearance of molecular techniques the recovery of viruses has dramatically increased. Nowadays virologists can quickly discriminate virological families and related viruses from emerging viruses and consequently identify novel viruses. Many new respiratory viruses have been identified in children in the past 15 years. In this review we shortly discuss novel respiratory viruses and their pathogenic role in pediatric respiratory disease. Advantages and drawbacks of the technique and our current knowledge will be discussed. We will conclude this review with a general discussion on the future role of molecular diagnostic virology in the clinic.

16.1 Introduction

16.1.1 General (Respiratory Viruses in Pediatrics)

Acute respiratory viral infections (ARVI's) are the most common infectious disease in humans. They occur more frequently in children than in adults (6.1 episodes per year under the age of 1, 3–6 episodes per year between the age of 1–5 and 2.4 episodes per year between the age of 15–19). Disease severity depends on age, underlying condition and type of virus. ARVIs account for huge numbers of doctor's visits and days lost from work and school. They are a leading cause of global mortality and morbidity in children. Moreover, respiratory viral infections are an important driver of unnecessary usage of antibiotics. Unfortunately prevention and treatment of the majority of respiratory virus infections is not possible with the exception of influenza [1].

Although much research has been done on the epidemiology and burden of viral respiratory tract infections the size of the problem is underestimated. Due to the lack of routine testing for (multiple) viruses and the limitation that a majority of infected patients will not visit a doctor.

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16.1.2 General Introduction Molecular Techniques (Discovery Novel Viruses)

With the appearance of molecular techniques the recovery of viruses has dramatically increased. Before their use approximately 50–80 % of the viral tests remained negative [2]. Due to the introduction of PCR and the discovery of novel viruses this proportion decreased to 3–15 %. However, such recovery rates are largely dependent on the selection of the patient group [3–5]. The combination of high sensitivity, multiplex options and quantification was essential for some of the new insights in viral epidemiology. This could not have been achieved with conventional viral diagnostics such as culture and immunefluorescence assays (FDA).

The identification of respiratory viruses in a clinical context can also guide diagnostic and treatment strategies. Bonner et al. [6] revealed that a known viral aetiology of disease results in decreased use of additional tests such as X-rays or blood examination, shorter hospital admission and less frequent use of antibiotics. However, molecular diagnostics have also created new dilemmas. For example, the identification of respiratory viruses in asymptomatic children, the occurrence of many viral co-infections, concerns about the pathogenic capacity of certain viruses and the value of quantitative measurements.

16.1.3 General Introduction on Emerging Viral Diseases

Some of the advantages of the new genetic (e.g. sequencing) and molecular techniques became clear during outbreaks of novel emerging viruses. Emerging viruses can be classified as (1) previously unknown viruses or (2) previously known viruses that have significantly increased in incidence [7]. Nowadays virologists can quickly discriminate virological families and related viruses from emerging viruses and consequently identify novel viruses.

The introduction of molecular diagnostics in medical virology has led to the identification of many new respiratory viruses in children in the past 15 years (Table 16.1). However, the pathogenicity of these viruses is not always clear and the clinical relevance is often poorly understood. Fredricks and Relman proposed seven rules which are necessary to demonstrate the causative relationship between a virus and disease. These rules are based on Koch's postulates and were adapted for nucleic acid based detection methods, location of the pathogen and quantification (Table 16.2) [8, 9]. These rules can help to interpret research on the role of novel respiratory viruses in disease and guide future research. It should also be stated that the clinical relevance is in some cases apparent, without extensive research to fulfil all requirements.

In this review we briefly discuss novel respiratory viruses and their pathogenic role in pediatric respiratory disease. We will conclude this review with general discussion on the future role of molecular diagnostic virology in the clinic.

Table 16.1 Emerging viruses from the last 2 decades

Virus	Family	Year of discovery
Hendra-/NipahV	Paramyxovirus	1995
AIV's	Influenza virus	1997
hMPV	Paramyxovirus	2001
SARS-CoV	Coronavirus	2003
HCoV-NL63	Coronavirus	2004
HCoV-HKU1	Coronavirus	2005
HBoV	Parvovirus	2005
HPeV4	Parechovirus	2006
HPeV5	Parechovirus	2006
HPeV6	Parechovirus	2007
KIV/WUV	Polyomavirus	2007
H1N1V	Influenza virus	2009

16.2 Henipavirus (1994–1998)

16.2.1 Hendra Virus

The Hendra virus was first detected in a disease outbreak in 1994. It initially presented with a new respiratory disease in horses that was transmitted to two persons one of them died [10]. The virus belongs to the genus of *Henipavirus* within

Table 16.2 Koch's postulates adapted for viral infections [8, 9]

A nucleic acid sequence belonging to a putative pathogen should be present in most cases of an infectious disease. Microbial nucleic acids should be found preferentially in those organs or gross anatomic sites known to be diseased, and not in those organs that lack pathology
Fewer, or no, copy numbers of pathogen-associated nucleic acid sequences should occur in hosts or tissues without disease
With resolution of disease, the copy number of pathogen-associated nucleic acid sequences should decrease or become undetectable. With clinical relapse, the opposite should occur
When sequence detection predates disease, or sequence copy number correlates with severity of disease or pathology, the sequence-disease association is more likely to be a causal relationship
The nature of the microorganism inferred from the available sequence should be consistent with the known biological characteristics of that group of organisms
Tissue-sequence correlates should be sought at the cellular level: efforts should be made to demonstrate specific in situ hybridization of microbial sequence to areas of tissue pathology and to visible microorganisms or to areas where microorganisms are presumed to be located
These sequence-based forms of evidence for microbial causation should be reproducible

the family of the *Paramyxoviridae* family. It was initially named morbillivirus and later re-named Hendra virus (HeV) after the suburb where the outbreak occurred [11, 12]. The virus itself is not very contagious. It spreads through direct contact between horses or during intensive contact between humans and severely ill horses. The animal reservoir appears to be the Flying-fox, in this population the Hendra infection is largely subclinical. The breading season of Flying-foxes is a risk period for spread and the human risk group is defined as people with close and intensive contact with horses. Up till now fourteen outbreaks have been reported [13]. In five of these outbreaks humans were involved, resulting in five deaths. So far, only two persons survived an infection. The case fatality rate (CFR) is over 50 % [13]. HeV in humans causes, after an incubation period of 5–21 days, a severe influenza like disease (fever, myalgia and headache) which can progress to pneumonia, respiratory failure and death [10, 14]. An infection can also result in encephalitis with headache, fever and drowsiness. The encephalitis can occur after initial recovery from the illness. The Hendra virus genome is readily detected in several materials, e.g. blood, urine, nasal- and oropharyngeal swabs by RT-PCR. Next to this standard detection method the virus can be cultured in several cell lines, where it forms syncytia upon infection. ELISA serological tests are used for screening, however their diagnostic sensitivity is not yet established. Immunofluorescense assays and serum neutralization methods can also be used [13, 15].

16.2.2 Nipah Virus

In 1998 and 1999 two large outbreaks of respiratory disease in pigs and humans occurred. In Malaysia and Singapore 106 people died [16]. The causative agent had large similarities to the HeV and is the second member of the genus of *Henipavirus* within the *Paramyxoviridae* family. It was named Nipah virus (NiV) after the location of the first human case [17]. This virus had already caused respiratory disease in pigs until late 1996. Like HeV it shares the bat as a natural reservoir. NiV virus is very contagious among pigs and spreads through the respiratory route or directly by the transport of infected pigs. Initially humans became infected via direct contact with pigs, although food borne transmissions were also reported. Initially the case fatality rate was 38.5 % [14, 18]. Since the initial outbreak almost yearly new outbreaks emerged in Bangladesh and India causing fatal encephalitis in humans. Notably, the CFR's of these outbreaks increased to 92 %. Also the transmission changed: starting from pigs, soon cows could transmit the virus. Later human to human and nosocomial transmission was demonstrated [19–21]. It has been hypothesized that there were multiple introductions of viruses in the human population, explaining the unique genetic signature of isolated viruses nowadays [18]. These genetic differences may be the reason for the increase in CFR and differences in clinical manifestations

and transmission. The clinical manifestation of a Nipah infection differed per outbreak. Incubation periods differ from an average of 2 weeks in Malaysia to 1 week in Bangladesh. The infection can be asymptomatic, but often starts with influenza-like symptoms of fever, headache, myalgia, vomiting and sore throat. Patients can recover or develop signs of encephalitis or sometimes atypical pneumonia or acute respiratory distress. In severe cases the encephalitis includes the brain stem or progresses to a coma within 24–48 h [18, 19, 22]. Around 20 % of the cases are left with residual neurological symptoms, including personality changes. In comparison with outbreaks in Malaysia and Singapore the Bangladesh and Indian patients experienced more profound respiratory symptoms with case rates of 14, 27, 70 and 51 %, respectively [19]. A Nipah infection can be diagnosed in serum urine and cerebrospinal fluid (CSF) by RT-PCR [23]. Also culture in cell-lines, ELISA for anti-HeV IgG and IgM in serum and CSF, serum neutralization assays or immunofluorescence assays are used [18, 19, 24, 25].

Patients with both Hendra and Nipah virus infections are treated supportive, antiviral therapy is not effective [22]. Prevention is based on careful hygiene, quarantine and safe disposal of animal carcasses [13, 14]. Currently, there are no vaccines available. However, several therapeutic agents seem effective *in vitro* and in some animal models [18].

16.2.3 Avian Influenza Virus (1997)

The first cases of avian influenza virus (AIV) infection were reported in 1997 in Honk-Kong [26]. This influenza A (H5H1) originated completely from strains circulating in wild birds and poultry [27]. The avian influenza virus undergoes rapid genetic and antigenic evolution reflected by the occurrence of different clades with distinct phenotypes [28, 29]. The majority of human cases had direct contact with poultry or could be related to outbreaks in wild birds [20]. There is limited transmission from human to human, although some epidemiological studies suggest it is possible [30]. The median age of patients is around 18 years and the mortality rate is extremely high between the age of 10 and 19 years (61 %). Yearly H5N1 outbreaks in humans have been reported in Asia, Africa and Eurasia [31]. These epidemics are all related to outbreaks of avian influenza in wild birds or poultry during the colder seasons [32, 33]. The incubation period of H5N1 is estimated to be 2–7 days [34]. The disease typically manifests as a severe pneumonia which often progresses to respiratory failure and death within 10 days (case fatality rate up to 90 % in children). It appears that in children cases may occur without pneumonia. Detection of viral RNA by (RT-) PCR is the best method for the diagnosis of H5N1, preferably using throat swabs [35]. Because of genetic variably of the virus, primers need to up-dated frequently. The available immune-assays for detection of H5N1 are not sensitive enough for clinical purposes and cannot differentiate between human and avian subtypes of influenza A. Seroconversion after 2–3 weeks can be used to confirm H5N1 infection and can be used for epidemiological studies [35]. Early treatment with oseltamivir is recommended based on some evidence that it increase survival rates [36]. There are differences in susceptibility to oseltamivir between the different clades of H5N1 circulating in different parts of the world. Combination of oseltamivir with amantidine can be given if the circulating H5N1 is susceptible to both agents. Currently, it is possible to produce vaccines that inactivate H5 influenza A strains. However due to the circulation of different clades and the rapidly changing antigenicity of H5N1 the need for the development of a new vaccine remains [34, 37].

16.2.4 Metapneumovirus (2001)

The human metapneumovirus (hMPV) was first discovered in the Netherlands in 2001 from a databank of samples from children with respiratory tract infections [38]. hMPV belongs to the genus *metapneumovirus* within the family of *Paramyxoviridae*. It is related to respiratory

Table 16.3 Symptoms and diagnosis of hMPV mono infections in literature

Symptoms/diagnosis	Spread in literature[a]
Fever[b]	36–80
Cough	67–99
Rhinitis	72–90
Wheezing	10–73
Respiratory failure	8
Oxygen 90 %	32–85
Pharyngitis	24–66
Bronchitis	1–68
Bronchiolitis	11–51
Pneumonia	3–65
Otitis media	16

[a] Heikkinen et al. [121]; Aberle et al. [122]; Mullins et al. [123]; Chen et al. [124]; Manoha et al. [125]; Williams et al. [126]; Bosis et al. [127]
[b] Different definitions varying from >37.5 to >39 °C

syncytial virus, both belonging to the *pneumoviridae* sub-family. In both retrospective and prospective studies it has been shown that hMPV can be detected in 3.9–14.8 % of respiratory samples from children with respiratory disease [39]. This wide range reflects differences in the tested populations and the level of care. Co-infections with other viruses occur in 15–30 % [40–42]. hMPV is detected in up to 4 % of nasopharyngeal aspirates from healthy children, although percentages of less than 1 % are also frequently published [43]. Serological studies showed that all children by the age of 5 years had been in contact with the virus and that it has been circulating in the human population for over 50 years [38]. It has a seasonal occurrence with a peak incidence just after the influenza and RSV season [43]. Spread is thought to be via direct or close contact with respiratory secretions from an infected person with an incubation period of 3–5 days. Re-infections occur frequently in children, although symptoms are less severe [44]. Symptoms associated with hMPV infections are comparable with RSV (see Table 16.3). hMPV infections are, after RSV, the most frequent cause of bronchiolitis in young children and account for 5–15 % of all hospital admissions [45]. Hospitalization rates are highest among 6–12 month old children, remarkably older than for RSV [46]. There is an association between severe hMPV infection (bronchiolitis) and the development of wheezing in childhood [47].

hMPV can only be cultured in specific cell lines under specific conditions and is time consuming; therefore it has no role in a clinical setting. Real time PCR is the most sensitive test for hMPV detection in NPA and swabs [48] and is therefore the common method in clinical and research settings. RT-PCR also provides semi-quantitative information of the viral load (Ct value), which can be used to monitor treatment in a research setting [49]. Immunofluorescence assays are available for rapid detection of the virus in respiratory specimens; however, these tests are less sensitive than RT-PCR. Serology for hMPV has little additive value in the clinic because most children are seropositive in early childhood. Currently no vaccines against hMPV are available, though several candidates are being pursued [45]. Ribavirin, antiviral therapy, is effective *in vitro* against hMPV, though clinical data are sparce. Currently new therapies such as fusion inhibitors and siRNA's are being tested in murine models [45].

16.2.5 Coronavirus (2003–2005)

Human corona viruses related to respiratory disease, 229E and OC43, have been known since the 1930s. They were recognized as the second most common cause of the common cold in humans [50–52]. A new strain of human coronavirus was identified in 2004 from a respiratory sample of a 7 month old infant with bronchiolitis and named NL63 (HCoV-NL63) [53]. The HCoV-NL63 belongs to the genus Coronavirus within the family of Coronavidae. In retrospective cohort studies HCoV-NL63 have been identified in 1.7–9.3 % of respiratory samples from children with respiratory symptoms and occurs worldwide [39]. The virus is often found in combination with other respiratory viruses (57 %) [54, 55]. Peak incidence is found in the winter months and the incubation period is estimated

2–5 days [56, 57]. HCoV-NL63 is associated with mild upper respiratory tract symptoms and less frequent with severe lower respiratory tract symptoms such as bronchiolitis [58]. Some studies have reported an association with croup [55, 59]. HCoV-NL can be detected in respiratory specimens by RT-PCR which is the first choice for diagnosis. Immunoassays are available for rapid detection and distinction of different HCoV strains [60]. Different cell-lines are permissive for viral culture and used in a research setting. Currently no anti-viral treatment against HCoViNL63 is available, although several inhibiting compounds have been identified [61].

A second novel human coronavirus was identified in 2005 in a 71-year-old man with pneumonia in China and named HKU1 after the Hong Kong University where it was found [62]. In a retrospective cohort studies the HCoV-HKU1 was identified in 1–3.1 % of respiratory samples in which no other virus was detected, from children with upper and lower respiratory symptoms [39] with a higher incidence in children younger than 6 months. The peak incidence of HCoV-HKU1 is in spring, early summer and winter with an incubation period of 2 days [63]. HCoV-HKU1 is mainly associated with upper respiratory tract symptoms in children and occasionally with pneumonia and bronchiolitis [64]. The first choice of assay for detection in respiratory specimens is RT-PCR. Coronaviruses exhibit substantial genetic variability hampering the development of pancorona primers and therefore specific primers for each strain have to be used [65]. There is no specific anti-viral therapy available against HCoV-HKU1.

16.2.6 Human Bocavirus (2005)

Human bocavirus (HBoV) belongs to the genus *Bocavirus* within the family *Parvoviridae* (and is closely related to the bovine parvovirus and canine minute virus). This virus was identified in 2005 by nucleic acid amplification (PCR) in respiratory tract specimens from Swedish children with lower respiratory tract infections [66]. In this study HBoV was detected in 3.1 % of hospitalized children below the age of three. Other studies detected HBoV in 3–19 % of children with respiratory symptoms depending on the sample type used (NPA and BAL *higher*, nasal swab *lower*) [67] and the age of the patient (*higher* in younger children) [68]. However, HBoV is frequently found in asymptomatic children (up to 40 %) or in combination with other viruses (up to 80 %) in symptomatic children [39]. Based on these findings it is still unclear whether HBoV has a pathogenic role in respiratory disease. One study performed in a PICU suggests that the viral load (high titres) of HBoV may indicate a pathogenic role in (severe) respiratory disease [69]. HBoV has been associated with wheezing in asthmatic children [70]. In general HBoV infection is marked by relatively mild symptoms of the upper respiratory tract such as cough, rhinorrhea and fever. In rare cases it has been associated with lower respiratory tract infection and even respiratory insufficiency [71]. Detection of HBoV is by RT-PCR and the virus can be detected in respiratory as well as gastrointestinal specimens [72]. Diagnostic seroresponses can be used to establish the specific immune response against HBoV during infection, although the clinical relevance is unclear [73]. HBoV can only be cultured on ciliated primary human epithelial cell-lines, and therefore viral isolation is only used in experimental settings [74]. Treatment of HBoV infections is mainly supportive and no specific anti-viral treatment against HBoV is available. Currently there is not enough epidemiological evidence to drive vaccine development against HBoV.

16.2.7 Parechovirus (2006–2007)

Human parechoviruses (HPeVs) belong to the genus *Parechovirus* of the family *Picornaviridae*. The first HPeVs, serotype 1 and 2, were identified 50 years ago during a summer diarrhoea outbreak in American children [75]. With the introduction of molecular techniques many new serotypes of HPeVs have been identified in the past 15 years in the stool or NPA of children with gastrointestinal and respiratory disease, and in the cerebrospinal

Table 16.4 HPeV, discovery and clinical associations

HPeV type	Known since	Clinical associations
HPeV1	1956	Mild gastrointestinal and respiratory symptoms, bronchiolitis, pneumonitis, otitis media. Encephalitis, paralysis, myocarditis
HPeV2	1956	Mild gastrointestinal and respiratory symptoms, (rare) neonatal sepsis, meningitis, encephalitis
HpeV3	2004	Neonatal sepsis, meningitis, encephalitis (transient paralysis)
HPeV4	2006	Fever, mild gastrointestinal and respiratory symptoms
HPeV5	2006	Fever, mild gastrointestinal and respiratory symptoms (sepsis, Reye's syndrome)
HPeV6	2007	Fever, mild gastrointestinal and respiratory symptoms (paralysis, Reye's syndrome)
HPeV8	2009	Enteritis
HPeV10	2010	Gastro-enteritis

fluid of children with meningitis and sepsis-like illness (see Table 16.4) [76–79]. Every HPeV serotype has its specific epidemiological and clinical features. All HPeVs infections are very common in children under the age of 1 year and most data are available on HPeV1 and HPeV3 [80]. The median age of infection with HPeV1 is 6.6 months, whereas HPeV3 infections occur at a younger age of 1.3 months. There is also seasonal variability in occurrence, HPeV1 in late summer and early winter season, and HBeV3 mostly in summer. HBeV serotype 5 and 6 have also been associated with respiratory tract symptoms [81–84].

Most HPeVs have are common causes of asymptomatic infection in early childhood and are often found in combination with other viruses, so that the relation with respiratory disease is hard to establish [80]. While the association of HPeV3 with encephalitis, meningitis and neonatal sepsis is widely accepted [85], for most other serotypes the relationship with disease and specific symptoms is less clear (see Table 16.4) [86].

A viral neutralisation assay or culture are time-consuming and not suitable for severe disease such as sepsis and meningitis. Detection by RT-PCR is only available for HPeV1-3 [87]. Currently amplification and nucleotide sequencing is used to identify specific genotypes in a research setting. The specific antibody response can be used to demonstrate involvement of HPeV in disease if the virus itself cannot be detected. No antiviral treatment against parechoviruses is currently available and only supportive care is given.

16.2.8 Polyomavirus (2007)

In 2007 two new members of the *Polyomaviridae* family were discovered in samples of patients with respiratory disease. The first of these new polyomaviruses was identified during a large scale molecular virus screening project in respiratory samples from children and named after the Karolisnka institute where it was discovered (KIV) [88]. The second was identified in a nasopharyngeal aspirate of a 3-year-old child with pneumonia and named Washington University virus (WUV) [89]. Seroprevalence studies show KI in 66 % and WU in 79 % of paediatric sera [46, 90]. The virus has been detected in 1–5 % respiratory samples worldwide in respiratory samples of young symptomatic children [91–93]. However, in 70–80 % of the cases there was a co-infection with other respiratory viruses, and KIV and WUV have been described in asymptomatic HSCT patients [94]. Based on these results it is difficult to assign symptoms and pathogenicity to both of them and more epidemiological evidence is needed. In most studies the viruses have been associated with both upper and lower respiratory tract infections in children. Detection of WUV and KIV in respiratory samples can be undertaken by RT-PCR. Thus far there is no indication for treatment of either of these viruses nor vaccine development.

16.2.9 Influenza A H1N1 Virus (2009)

In late march 2009 a novel influenza A (H1N1) virus was identified in America. This virus was subsequently recognised as the cause of an outbreak of respiratory illness in Mexico [95]. The

Table 16.5 Symptoms of H1N1

Presentation	Literature[a] (%)
Fever (>38 °C)	81–94
Cough	69–82
Gastro-intestinal symptoms	8–32
Rhinorrhea	31–62
Diarrhea	8–23
Wheezing	12–25

[a] Libster et al. [97], Hackett et al. [128], Jain et al. [129]

novel flu virus showed reassortment of swine, avian and human strains, and appeared to be very infectious between humans [96]. After the initial detection several other countries reported H1N1 infections. In June 2009 the WHO declared a pandemic with spread over at least two continents. At the start of the pandemic the virus appeared to be very virulent with a high mortality rate, especially in young adults and children [97, 98]. However, in the Northern Hemisphere the virus behaved more like a seasonal influenza virus. H1N1 disease had the highest attack rate in young children causing relatively mild disease [99]. The pH1N1 was able to outcompete the seasonal flu so that, in the influenza season 2009–2010, over 99 % of the influenza positive isolates in Europe and America were pandemic H1N1 influenza A [101].

In general the symptoms resembled those of other winter viruses: fever, cough, sore throat, myalgia and headache. Symptoms at presentation for hospitalised patients are shown in Table 16.5. Spread occurs up to 8 days after the start of symptoms although this may be prolonged in immunocompromised patients and children [102].

H1N1 infection can be diagnosed by RT-PCR on respiratory samples and this appears to be the most sensitive method. In case of high suspicion of H1N1 infection with a negative PCR result, the virus can be cultured or infection proven by documenting seroconversion [103–105].

During the pandemic of H1N1 were treated with oseltamivir (Tamiflu®) and zanamivir. This treatment reduced the duration of symptoms, the occurrence of otitis media and progression into severe disease, especially when administered early in the course of disease [106]. Also the prophylactic use of anti viral agents is effective in reducing the occurrence of H1N1 infections in exposed individuals. However, oseltamivir and multi drug resistant viruses are emerging [107]. In several countries children have been vaccinated [108, 109]. H1N1 vaccination induced an effective and long lasting humoral immune response [108, 109]. The vaccine seemed to reduce the risk of infection and decreased severity of disease in children, however because of the rapid spread of the H1N1 pandemic most people were vaccinated during the pandemic making efficacy studies complex [111, 112].

16.3 Discussion on Molecular Diagnostics of Respiratory Viruses and Their Clinical Use

In this review we have discussed newly identified and emerging viruses from the past 2 decades. These viruses could be subdivided in three categories, based on the evidence for their pathogenicity in respiratory disease in children. First, emerging viruses causing epidemics with high mortality, such as AIV, Hendra and Nipah virus, were clearly associated with a pathogenic role in disease. These epidemic-causing viruses are often of zoonotic origin (transmission from animals to humans). The second group comprises viruses that fulfil the modified Koch's postulates [8, 9]. Most novel respiratory viruses are not completely characterised according to the postulates due to the extensive and costly research needed to achieve this. In this perspective hMPV is unique among the recently discovered respiratory viruses, because all criteria have been fulfilled [111]. Third are viruses that were found during screening for new respiratory viruses in respiratory samples with molecular techniques, such as human bocavirus, the novel polyomaviruses, parechoviruses and some coronaviruses. For most of these viruses their pathogenic role as an important respiratory pathogen is less clear. Although these viruses are present in respiratory

samples of children with respiratory disease however, they are also often present in asymptomatic children or found in combination with other viruses. Many studies were performed retrospectively, or without the proper control cohorts of asymptomatic children. In epidemiological studies based on seroconversion it is apparent that a first encounter with these viruses occurs early in childhood without (severe) respiratory tract infections. Especially in this last category of viruses, in which the association with respiratory disease is less clear, large prospective epidemiological studies are needed to further specify the pathogenicity and health burden of these viruses in children.

The highly sensitive molecular techniques for identification and detection of novel viruses are a powerful tool for epidemiological studies, especially when used in multiplex platforms. Their ability to quantify the viral burden in infection may be used as additional information in determining the role of a virus in respiratory disease. For some viruses a positive correlation between viral load and disease severity is described [69, 115]. However whether viral load correlates with disease severity in general remains a point of debate. Viral load appears to be lower in viral-viral co-infection compared to viral-mono-infections, the mechanism behind this and the clinical relevance requries further investigation [116, 117]. Studies show that viral load decreases during the course of disease, and this can be used as marker for the therapeutic effect of anti-viral compounds. A drawback of the high sensitivity of molecular diagnostics is that PCR signals remain positive after recovery from an illness, sometimes even for several weeks. Because young children have frequent viral infections of the upper respiratory tract, the value of a positive PCR test can be limited.

Interaction of viruses with bacteria present in the nasopharynx can result in enhanced disease severity. This is well known for influenza and *Streptococcus pneumoniae*, and other respiratory bacteria [118]. How other (novel) respiratory viruses interact with bacteria and how this leads to enhanced disease is less well known. In studying the pathogenicity of viruses these interactions should be taken into account and implemented in new epidemiological studies. In this context 'old' viruses, like rhinovirus, can be seen in a new perspective and the causality with severe respiratory disease should be re-evaluated [118–120].

The introduction of molecular detection of viruses has led to the discovery of many new human respiratory viruses and improvement in diagnostics. Novel molecular techniques, like sequencing of the complete virome, will offer new insight in viral infections but also new challenges in proving causality in human disease.

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