Chapter 5
Respiratory Infections

Michael D. Nissen, Stephen B. Lambert, David M. Whiley, and Theo P. Sloots

Abstract Until recently, conventional culture techniques and immunofluorecence assays were considered the gold standard for the detection of respiratory viruses, even though results are mostly available too late or lacked specificity and sensitivity. These methods are now widely replaced with appropriate DNA- and RNA-based amplification techniques, in particular real time PCR amplification, for the detection of an extended number of agents responsible for acute respiratory infections. Real-time PCR offers rapid results, efficiencies in work flow and a reduced risk of false positive results due to contamination. As a result, better patient management or reduction of unnecessary antibiotic administration will be possible leading to enhanced efficiencies in health care. In applying molecular methods to diagnostic use, the laboratory can optimise its diagnostic strategy by applying a combination of real-time amplification tests for respiratory viruses and the non-viral respiratory bacterial pathogens. However this must be done within a context of resource availability, technical expertise available and clinical utility. It seems certain that molecular microbiology will continue to develop, leading to further applications in diagnostic technology, thereby improving our understanding of disease processes and enhancing our knowledge of the pathogens responsible.

Keywords Respiratory infection · Diagnosis · Virus · Bacteria · Fungi · Clinical · Pathogenesis · Epidemiology · Emerging viruses

5.1 Introduction

Acute respiratory infections (ARIs) continue to be the leading cause of acute illnesses worldwide and remain the most important cause of mortality in infants and young children.

T.P. Sloots (✉)
Queensland Paediatric Infectious Diseases Laboratory, Sir Albert Sakzewski Virus Research Centre, Queensland Children's Medical Research Institute, Children's Health Service District, Brisbane, QLD 4029, Australia
e-mail: t.sloots@uq.edu.au

M. Schuller et al. (eds.), *PCR for Clinical Microbiology*,
DOI 10.1007/978-90-481-9039-3_5, © Springer Science+Business Media B.V. 2010

They account for about two million deaths each year [22] and rank first among causes of disability-adjusted life-years (DALYs) lost in developing countries (94.6 million, 6.3% of total [43]). The populations most at risk for developing a fatal respiratory disease are the very young, the elderly, and the immunocompromised.

While upper respiratory infections (URIs) are very frequent but seldom life-threatening, lower respiratory infections (LRIs) are responsible for more severe illnesses such as influenza, pneumonia, tuberculosis, and bronchiolitis that are the leading contributors to ARIs' mortality (Fig. 5.1). Pneumonia, with a global burden of 5,000 childhood deaths every day, is a tangible threat that needs to be dealt with accordingly.

The incidence of ARIs in children aged younger than 5 years is estimated to be 0.29 and 0.05 episodes per child-year in developing and industrialized countries, respectively, which translates into 151 million and 5 million new episodes each year, respectively [28]. Most cases occur in India (43 million), China (21 million), Pakistan (10 million), Bangladesh, Indonesia and Nigeria (56 million each). Pneumonia is responsible for about 21% of all deaths in children aged younger than 5 years, leading to estimates that of every 1,000 children born alive, 12–20 die from pneumonia before their fifth birthday [43].

The main aetiological agents responsible for ARIs in children include *Streptococcus pneumoniae, Haemophilus influenzae* type b (Hib), *Staphylococcus*

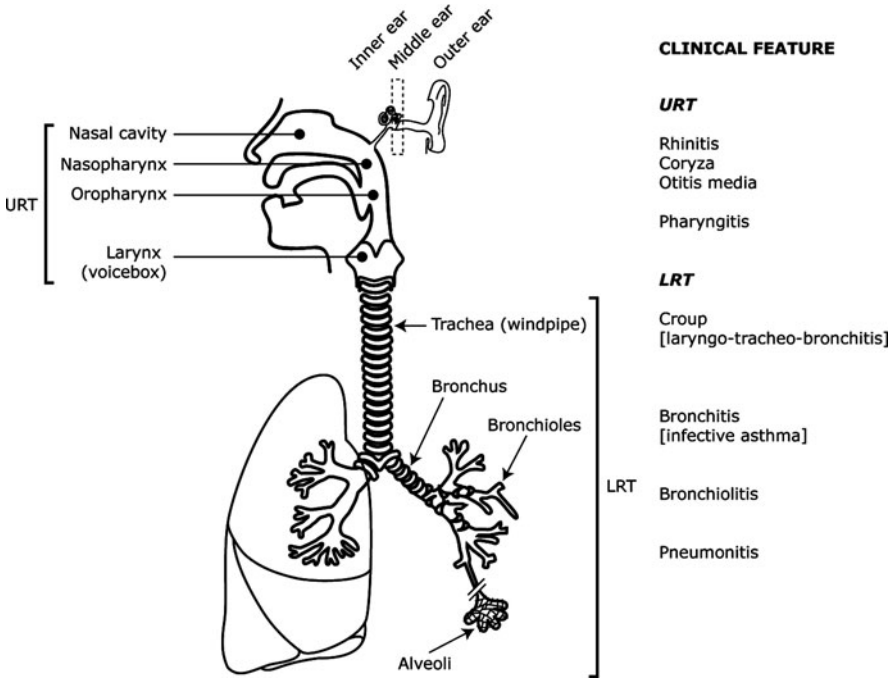

Fig. 5.1 A schematic representation of the human respiratory tract. The upper and lower respiratory tract and components of the ear are indicated together with other major respiratory sites

aureus and other bacterial species, respiratory syncytial virus (RSV), measles virus, human parainfluenza viruses type 1, 2, and 3 (PIV-1, PIV-2 and PIV-3), influenza virus (INF) and varicella virus.

5.2 Clinical Aspects and Epidemiology

5.2.1 Clinical Presentation

A wide variety of well known and newly identified agents cause respiratory illness and disease in humans. It is not possible to differentiate with certainty the aetiological agent in an infection based on clinical symptoms alone. In pre-school aged children, for example, illnesses due to respiratory viruses have seasonal variations and differences in the presence of fever and other symptoms, the likelihood of household transmission, and the impact they have in terms of medical visits and disruption to family life. But none of these features in isolation or combination is sufficiently specific to link illness to a pathogen with certainty.

Diagnosis in individual mild illnesses may not alter management, but can prevent unnecessary hospitalisation, antibiotic therapy, or further invasive investigation. Laboratory confirmation of the cause of infections has been made more sensitive and rapid through the use of PCR technology. PCR has taught us that the constellation and severity of symptoms can cluster with particular infectious agents. For example, recent findings from the New Vaccine Surveillance Network in the United States show that despite respiratory syncytial virus (RSV), parainfluenza viruses (PIVs), and human coronaviruses (HCoVs) all being common in early childhood; RSV and PIVs are more common causes of hospital admission with acute febrile and respiratory illness than HCoVs [33, 41]. Despite such clustering, in individual illnesses it can be said that even viruses more typically associated with severe childhood illness can cause milder symptoms, modified by immune or possibly genetic factors, and that severe disease, whilst more commonly caused by a small group of well-studied viruses, can result from infection due to any virus. Viruses that are typically considered to cause infrequent or mild disease, such as influenza C virus and PIV-4, may cause more significant illness in vulnerable populations, including the young and immunocompromised.

5.2.2 Pathogenesis (Transmission, Incubation, Site of Infection)

Influenza, the most studied of respiratory viruses, provides broad insights for other common myxovirus and paramyxovirus respiratory agents. A review of healthy adult human volunteer studies showed that viral shedding increased sharply between 0.5 and 1 day after influenza virus challenge, peaking on day two; shedding can be detected 24 to 28 h before clinical onset, and has a mean duration of 4.8 days; two-thirds of subjects had symptomatic infection, and total symptom scores peaked on day three [11]. The natural history of infection may differ in the elderly and

children. For example, pre-symptomatic influenza virus shedding has been seen for 6 days before clinical onset and mean duration of virus isolation from hospitalised children not receiving an antiviral was 6.8 days [29].

Respiratory viruses can be transmitted through a number of modes: direct contact and fomites, large droplet, and airborne small particles. The importance of each of these modes depends on the virus in question, the site of infection, and the environment. For example, the eyes and nose appear to be much more important routes of infection for RSV than the mouth.

5.2.3 Epidemiology (Frequency, Seasonality, Age Groups)

Modern molecular methods have resulted in the identification of previously unknown viruses from specimens collected from the respiratory tract. Testing for new viruses along with known viruses, including rhinoviruses [6] by PCR, is filling the diagnostic void in respiratory illness and infection, and has improved our understanding of the epidemiology of such illnesses. In all but tropical climates there are a group of respiratory viruses that occur more frequently in the non-summer months, often peaking in winter; these viruses include influenza viruses, RSV, human metapneumovirus (HMPV), PIVs, and HCoVs (Fig. 5.2). Human rhinoviruses (HRVs) are the most commonly identified group of viruses in both community-managed and more severe respiratory illness in children and older age-groups, having a year round presence but being more common in the spring and autumn months [6]. Whilst it is clear rhinoviruses are a major pathogenic group, there is still uncertainty about the predictive value of a positive molecular test for a picornavirus, particularly from children. In tropical settings, influenza and other respiratory viruses can have a high background year-long presence [38].

Respiratory viruses circulate freely in all populations, but moderate to severe illness tends to disproportionately affect certain groups. Infections due to common viruses that result in disease severe enough to warrant laboratory testing, notification, or hospitalisation occur in the young, the very old, or both, such as with RSV and influenza [9, 14].

5.3 Commonly Recognised Respiratory Agents

5.3.1 Respiratory Disease Due to Viruses

In spite of the inclusion of the live attenuated measles vaccine in the Expanded Program of Immunization (EPI), measles virus was still responsible in 2002 for some 213,000 deaths worldwide, essentially due to insufficient vaccine coverage [27] . The situation has fortunately been substantially improved lately, but the leading cause of serious respiratory illness in young children is respiratory syncytial virus (RSV), the agent of infantile bronchiolitis, which is associated with substantial morbidity and mortality [21]. Parainfluenza viruses (PIV-1, PIV-2 and PIV-3),

especially PIV-3, are second in incidence immediately after RSV. All children by the age of 2 years have had at least one episode of PIV and/or RSV illness. In addition, both viruses can cause severe disease in the elderly, especially in patients with a chronic respiratory or cardiac condition [18]. Although the disease burden due to these pathogens has not been accurately quantified in developing countries, extrapolation from known figures in industrialised countries, such as 125,000 reported cases of RSV per year in the USA, leads to the impressive global estimates of 64 million cases and 160,000 deaths per year from RSV infection worldwide. RSV was identified in 15–40% of pneumonia or bronchiolitis cases admitted to hospital in developing countries, followed by influenza viruses, parainfluenza viruses, human metapneumovirus and adenovirus [40]. The elderly also are at risk for severe RSV disease, and 14,000–60,000 RSV-related hospitalisations of the elderly are reported to occur annually in the USA [14].

Human metapneumovirus, a member of the *Paramyxoviridae*, is a recognised cause of a large fraction of severe ARIs in infant, elderly and immunocompromised populations [15]. Other viruses that cause respiratory infections are coronaviruses, adenoviruses and rhinoviruses. Recently discovered coronaviruses HCoV-HKU1 and HCoV-NL63 are significant pathogens that contribute to the hospitalisation of children for ARI [24, 35]. Among other members of the *Coronaviridae* are human coronaviruses HCoV-229E and HCoV-OC43, agents of the common cold. Another recently identified coronavirus is that of the severe acute respiratory syndrome (SARS), SARS-CoV, which emerged in southern China in late 2002 and spread in the spring of 2003 to some 30 countries within Asia, Europe and North America.

In the elderly, influenza-related pneumonia remains a leading cause of infectious disease-related deaths. The threat of an avian influenza pandemic has been looming ever since the emergence in 1997 in Hong Kong of the H5N1 avian influenza virus,

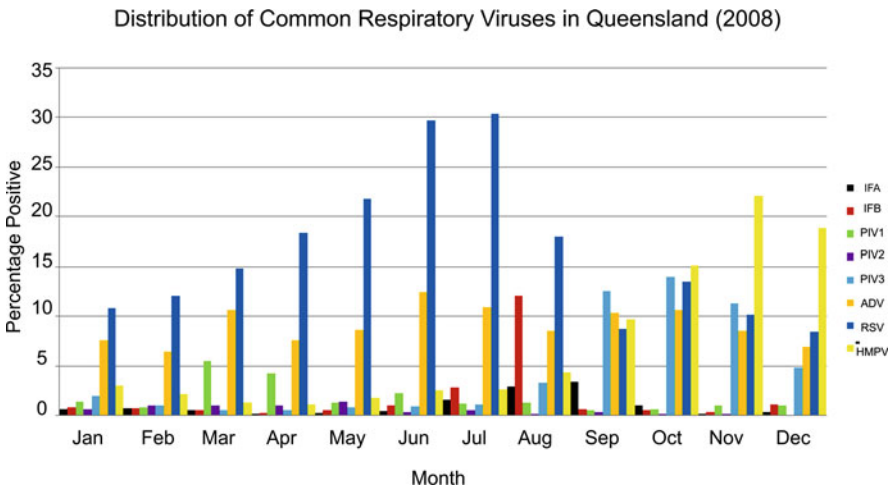

Fig. 5.2 Seasonal distribution of major respiratory viruses in Queensland, Australia for 2008

especially since the reappearance of human cases in 2003–2004. The new H5N1 variant is highly pathogenic for poultry and wild birds and can lethally infect cats and humans. At this time, however, it still is not possible to predict which virus is going to eventually cause a pandemic and when it is going to happen, but the preparation of pandemic influenza vaccines is being actively pursued, generating broad new knowledge on how to improve seasonal influenza vaccine immunogenicity. This has proven to be of the utmost importance with the recent emergence of influenza virus A H1N1 (2009) ("Human Swine Influenza") in Mexico and the USA in 2009.

Regarding influenza virus, the average global burden of inter-pandemic influenza may be on the order of 1 billion cases per year, leading to 300,000–500,000 deaths worldwide. However, the substantial reduction in ARI mortality observed in developing countries that have implemented simple case management, including provision of antibiotics to children with ARI, suggests that bacterial pneumonia contributes to a large proportion of deaths in these populations. Available data suggest that dual infections with viral and bacterial pathogens may be quite common, as seen by the fact that, in the industrialized world, epidemics of RSV and/or influenza coincide with epidemics of *S.pneumoniae* year after year [32]. While influenza virus is the most commonly met pathogen in this context, other respiratory viruses, including RSV, measles virus, parainfluenza viruses, or adenoviruses may also predispose to secondary bacterial infections. Several different bacterial species may be implicated, including *H. influenzae*, *Staphylococcus aureus, Streptococcus pyogenes, Mycoplasma pneumoniae*, and, most importantly of all, *S. pneumoniae* [25]. Half or more of the flu-associated mortality in the 1918–1919 Spanish Flu epidemic is believed to have resulted from pneumococcal super-infections.

The same is true for developing countries. As an example, the observation was made in South Africa that children vaccinated with the 7-valent conjugate pneumococcal vaccine showed 31% reduction in virus-associated pneumonias requiring hospitalisation, strongly emphasising the presumed importance of dual infections involving *S. pneumonia* [13]. Dual infection seems to increase the severity of the disease and to result in higher mortality. This might be due to inhibition of pulmonary antibacterial defenses during recovery from viral infectiions.

5.3.2 Respiratory Tract Infections Due to Bacteria

Streptococcus pneumoniae (pneumococcus) was identified in 30–50% of bacterial pneumonia cases in developing countries in the 1990s, followed by *Haemophilus influenzae* type b (Hib; 10–30% of cases), then *Staphylococcus aureus* and *Klebsiella pneumoniae* [28]. Non-typable *H. influenzae* (NTHI), and non-typhoid *Salmonella spp*. have also been implicated in some but not all studies. Other organisms, such as *Mycoplasma pneumoniae,* Chlamydia spp., Pseudomonas spp. and *Escherichia coli* also can cause pneumonia. The most common syndromes associated with *M. pneumoniae* infections are acute bronchitis, pharyngitis and otitis, but 10% of infected children develop pneumonia [39].

The introduction of Hib conjugate vaccines has resulted in a truly remarkable decline in Hib disease where the vaccine has been introduced. However, the vaccine is not yet routinely made available to a majority of children worldwide. As a result, 400,000 deaths are still estimated to occur from Hib disease each year [12]. In view of their safety and remarkable efficacy, the WHO has recommended the global implementation of the Hib conjugate vaccines.

S. pneumoniae is estimated to cause more than one-third of the 2 million deaths due to ARIs, especially in developing countries where the bacterium is one of the most important bacterial pathogens of infancy and early childhood [45]. Virtually every child in the world is colonised with one or more strains of pneumococcus and becomes a nasopharyngeal carrier during their first few years of life. Many children will go on to develop otitis media, and a few will eventually develop invasive pneumococcal disease including bacteraemic pneumonia and/or meningitis. The introduction of the conjugate pneumococcal vaccine in routine infant immunization should have a major impact on pneumonia in children less than 5 years of age worldwide, as already documented in the USA [26].

Tuberculosis (TB) continues to be a leading cause of deaths worldwide, with an estimated one third of humanity infected and about 1.7 million deaths each year, a global toll of 4,650 lives daily. The emergence of *Mycobacterium tuberculosis* (Mtb) strains carrying drug-resistance mutations against first-line dugs (MDR-TB) and, more recently, against both first- and second-line drugs (XDR-TB), shows that it will most probably be impossible to contain the TB pandemic with drugs alone. More than one hundred new TB vaccine candidates have been tested in animal models and some have moved into clinical trials. Testing such a wide variety of vaccine types using different strategies will obviously require time and a lot of coordination, especially as surrogate markers of protection still remain mostly unknown at this time.

Finally, it should be emphasized that nosocomial or hospital-acquired pneumonia is a major public health problem: pneumonia is the second most common type of all nosocomial infections, with an associated case fatality rate of 20–50%.

5.3.3 Respiratory Infections Due to Fungi

Infectious fungal respiratory diseases can be divided into those that occur opportunistically in immunosuppressed patients and those that occur in generally healthy individuals. Fungi which affect immunosuppressed individuals are frequently *Pneumocystis jiroveci*, and species of *Aspergillus* and *Candida* as well as *Cryptococcus neoformans* [5], while organisms such as *Histoplasma capsulatum, Coccidioides immitis* and *Blastomyces dermatitidis* are frequent pathogens in healthy individuals in certain endemic regions.

The causative fungi of respiratory infections vary with the population selected and the geographical region. The majority of fungal infections in lung-transplant recipients involve *Aspergillus* spp., followed by *Candida, Pneumocystis, Cryptococcus*, geographically restricted agents and newly emerging fungal pathogens. *Aspergillus* infection remains the main fungal complication in

lung-transplantation recipients. There are various fungal agents responsible for pulmonary fungal infection in patients with haematological malignancies, but *Aspergillus* spp. and other molds such as zygomycetes or *Fusarium* spp. represent the most frequently isolated microorganisms [19]. Less commonly, pneumonia could be due to *Candida* spp., *Cryptococcus* spp. or *Pneumocystis jirovecii* [17, 20]. Although invasive aspergillosis is an uncommon complication of haematopoietic stem cell transplants (HSCT) and solid organ transplants (SOT), it continues to be associated with poor outcomes [5]. Invasive pulmonary aspergillosis is rare in patients with chronic obstructive lung disease and is commonly associated with high doses of corticosteroids and multiple broad-spectrum antibiotics [19].

Candida infection is predominant in patients with non-haematologic malignant tumours and in non-lung SOT recipients. *Candida albicans* and *Candida parapsilosis* were the predominant isolates of pulmonary candidiasis in ventilated preterm infants with a birth weight of less than 1,250 g; the incidence rate of pulmonary candidiasis during the first month of life was 8.6% (20/233 cases) [17]. In Intensive Care Units, *C. albicans* was also the most frequently isolated fungal species in all sites (68.9%). Isolation of fungi allowed a diagnosis of fungal infection in 121 patients (7.7%) [47].

Cryptococcus infection occurred in both immunosuppressed and immunocompetent individuals. Pulmonary cryptococcosis is usually the primary site of a disseminated or central-nervous-system cryptococcal infection that may be fatal [46]. Traditionally, capsule-deficient *Cryptococcus neoformans* was considered to have low virulence. However, a recent study showed that the presentations and outcomes did not differ significantly between patients with proven pulmonary cryptococcosis caused by capsule-deficient *Cr. neoformans* and six patients with pulmonary cryptococcosis caused by capsule-intact *Cr. Neoformans* [46].

The predisposing factors for fungal respiratory infections are increasing with the emergence of new immunosuppressive treatment. The increase of fungal associated respiratory tract infections may predominantly be attributed to the development of invasive diagnostic tools and the use of new methods for the identification of isolates, such as molecular techniques.

5.4 New Viruses Associated with the Respiratory Tract

Recent advances in molecular biology have greatly improved the detection of viral respiratory pathogens. Yet, even with the most sensitive molecular techniques, only 40–60% of infections are consistently diagnosed. This suggests that additional respiratory viruses are likely to exist. In fact, since 2001, seven previously undescribed viruses have been identified by analysis of clinical specimens from the human respiratory tract (Table 5.1).

These new viral agents were detected by novel molecular methods such as virus discovery based on cDNA-AFLP (amplified fragment length polymorphism) (VIDISCA), pan-viral DNA microarrays and high throughput sequencing [4]. More

Table 5.1 Newly recognized viruses in the human respiratory tract: a summary of distribution, clinical association and methods of discovery

Virus	Patient Group	Prevalence	Clinical signs	Method of discovery	Reference
HMPV	Children and the elderly	3–25%	Bronchiolitis, pneumonia, bronchitis, rhinorrhoea, cough, sore throat	Virus isolation, electron-microscopy, and random PCR	[34]
SARS CoV	All ages	Sporadic	Pneumonia	Virus isolation, electron-microscopy, and consensus coronavirus PCR	[23]
NL63 HKU1	Children and the elderly	1–10%	Bronchiolitis, pneumonia, rhinorrhoea, fever, cough, wheezing	Virus isolation, VIDISCA, consensus coronavirus PCR	[36, 44]
HBoV	Children	1–11%	Bronchiolitis, pneumonia, acute otitis media, asthma	Random PCR	[1]
KIV WUV	Children	1–7%	Bronchiolitis, pneumonia, cough	Random PCR	[2, 16]

broadly, the advent of these new technologies has greatly stimulated efforts to identify novel viruses in the respiratory tract and in other human disease states.

Of the viruses discovered over the last 7 years, human metapneumovirus (HMPV) and the newly emerging human coronaviruses (HCoV) are considered causative agents of respiratory disease. However, to date, SARS coronavirus has been restricted geographically and has only been associated with limited and sporadic outbreaks. Recently, human bocavirus (HBoV) and the new human polyomaviruses KIV, WUV and Merkel Cell polyomavirus (MCV) were detected in respiratory secretions, and although an association with the respiratory tract has been postulated, it still remains to be proven [2, 8, 16].

5.4.1 Human Metapneumovirus

HMPV infection is associated with a broad spectrum of clinical signs in patients of all age groups, and is the cause of upper and lower respiratory tract infection in infants and young children [30]. It is second only to RSV as a significant cause of bronchiolitis in early childhood and children are most likely to be hospitalised with severe disease. Studies have linked HMPV with acute otitis media and asthma exacerbations in children and with exacerbations of both asthma and chronic obstructive pulmonary disease (COPD) in adults. However, severe disease may occur in all patients with underlying medical conditions such as cardiopulmonary disease, the elderly and immunocompromised subjects. In these subjects the virus can cause prolonged and serious infections, particularly severe lung disease including fatality [10] (Fig. 5.3).

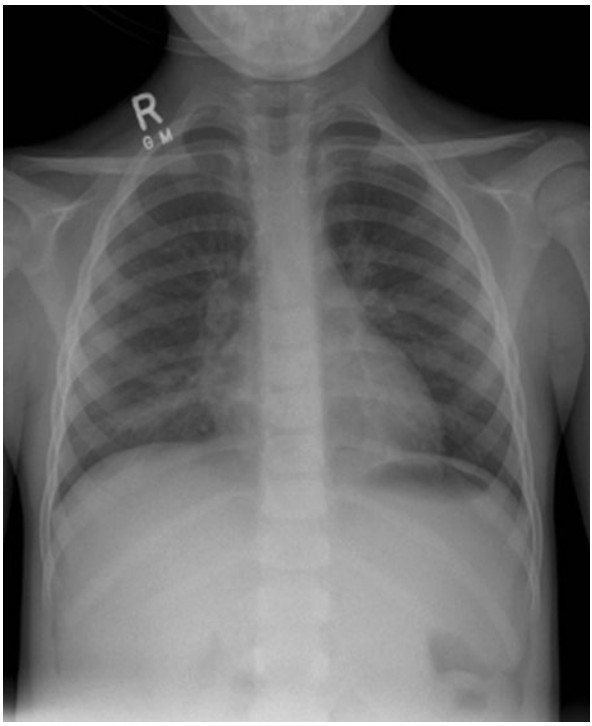

Fig. 5.3 Example of representative radiography showing features of RSV infection

5.4.2 SARS Coronavirus

The first reports of SARS coronavirus infection were published in 2003, and the causative agent was subsequently characterised as a novel human coronavirus [23]. The SARS epidemic was halted by a highly effective global public health response coordinated by the World Health Organization, and there is no further evidence that SARS CoV is currently circulating in humans. However, the SARS outbreak focused renewed attention on coronaviruses generally, resulting in the discovery of two more new human coronaviruses, NL63 and HKU1.

5.4.3 Human Coronaviruses NL63 and HKU1

HCoV-NL63 was first detected in 2004 in a child from The Netherlands with bronchiolitis, shown to be a cause of severe lower respiratory tract infection (LRTI) in young children, and an agent of laryngotracheitis (croup) [37].

HCoV-HKU1 was detected in 2005 in an adult with chronic pulmonary disease in Hong Kong [44] and was subsequently shown to be globally distributed. HKU1 infection presents with common respiratory symptoms as well as a more severe clinical presentation including bronchiolitis and pneumonia [24].

In healthy adults HCoV NL63 and HKU1 infections are generally not life threatening. This suggests that these coronaviruses, like 229E and OC43, only cause more-severe disease in young children, elderly persons, and the immunocompromised. They may be detected in 1 to 10% of patients with acute respiratory tract infections, and co-detection of these viruses with other respiratory viruses is common [37].

5.4.4 Human Bocavirus

Human bocavirus was first described in 2005 with a prevalence of 3.1% in Swedish children with LRTI [1] and subsequently in Australia with 5.2% prevalence in children with ARTI during winter [31]. Although a positive association of HBoV with ARTI was suggested, the results remain inconclusive, because a high prevalence of other respiratory viruses was found in HBoV-positive patients. To confirm the role of HBoV as a respiratory pathogen, more extensive studies including matched control populations need to be performed. One such study using control subjects [3] proposed HBoV as a cause of acute wheezing in children.

HBoV has been frequently detected in immunosuppressed adults but only rarely in immunocompetent adult subjects. However, it is uncertain if the presence of HBoV in these subjects is the result of re-infection, viral persistence or reactivation.

5.4.5 Novel Human Polyomaviruses KIV, WUV and MCV

Recently, three new human polyomaviruses, KIV, WUV and MCV were detected in specimens of patients with ARTI [2, 8, 16]. Allander et al. [2] reported a prevalence of 1% for KIV in nasopharyngeal aspirates (NPA) collected from a Swedish population and Gaynor et al. [16] showed a prevalence of 3% and 0.6% for WUV in respiratory samples from Australia and the USA respectively. More recently, although originally found in Merkel cell carcinoma, MCV was also found in 5.9% of NPA collected from Australian subjects with ARTI [8]. Since these first reports, KIV, WUV and MCV have been detected in a number of geographic locations, suggesting a global presence for these viruses.

One striking feature of early findings concerning KIV and WUV is their high rate of co-detection with other respiratory viruses. A co-detection rate of 74% has been observed for KIV and rates ranging from 68% to 79% for WUV [7]. So, even though an aetiological role in childhood respiratory disease has been proposed, it is difficult to assess a pathogenic role for these viruses unless observations are compared with those for matched control populations.

Further studies will need to be completed before the role of KIV and WUV as respiratory pathogens can be confirmed, and it remains possible that these viruses are not involved in respiratory disease, but that their presence in the respiratory tract simply reflects their mode of transmission.

5.5 Laboratory Diagnosis

Cheryl Bletchly

5.5.1 Respiratory Specimens and Transport

Laboratory diagnosis of respiratory virus infections requires specimens containing cells from the respiratory tract collected early in the clinical illness. The most appropriate specimens are NPAs and bronchoalveolar lavage (BAL). Where the generation of aerosols may pose an infection risk to collection personnel, nose and throat swabs are a viable alternative. Recent studies however, have shown that swabs are not as effective as NPAs for the detection of adenovirus and respiratory syncytial virus. Limited data is available suggesting that flocked swabs are superior to traditional swabs for detecting respiratory virus infections.

Collection swabs should be dry and not contain bacterial transport medium as these often contain substances that are inhibitory to PCR reactions.

In the case of influenza virus detection, the CDC recommends that Viral Transport Medium (VTM) is added to swabs to assist in virus preservation. If VTM is to be added to dry swabs, they should be vortexed to release cells in to the medium and transferred to a sterile vial for transportation to the laboratory. VTM cannot be added to swab receptacles as they will invariably leak in transit and pose an infection risk. All respiratory specimen types should be transported to the laboratory at 4 °C. Some NPAs and sputum samples received in the laboratory are very mucoid. These may either be diluted in VTM or digested with sputasol to facilitate the extraction process prior to molecular analysis.

It is preferable to use an extraction method that will extract both RNA and DNA with equal efficiency as additional testing for agents such as *Bordetella pertussis* and *Mycoplasma pneumoniae* are often requested if a viral cause cannot be found. Lung tissues are often collected at autopsy for either culture or molecular viral studies.

The specimen requirements for both molecular and culture based respiratory virus detection methods are similar and therefore laboratories can choose the detection method that suits their role.

All respiratory specimens must be handled with appropriate personal protection equipment as specified for PC2 laboratories and opened and aliquotted only in a Class II Biological Safety Cabinet. Specimens should be stored at 4°C until they are processed.

C. Bletchly (✉)
Molecular Diagnostic Unit, Microbiology Division, Pathology Queensland Central Laboratory, Brisbane, QLD 4029, Australia
e-mail: Cheryl_Bletchly@health.qld.gov.au

5.5.2 Traditional Methods

Traditional laboratory respiratory virus diagnosis involved virus culture with, or without immunofluorescence staining with specific antibodies. Culture is highly sensitive if the appropriate cell lines are utilised and once a virus is isolated it can be further characterised and amplified if required. Viral culture provides the added advantage that it will only detect infectious virus and not persistent nucleic acid. It has the disadvantages however of being expensive due to the requirement to maintain cells lines and tissue culture media and the requirement for expertise in sterile technique and interpretation of cytopathic effect (CPE) and fluorescence staining.

Direct Immunofluorescence Assay (DFA) of respiratory tract cells provides rapid results but does involve a series of manual manipulations and washes along with interpretation by highly skilled personnel (Fig. 5.4).

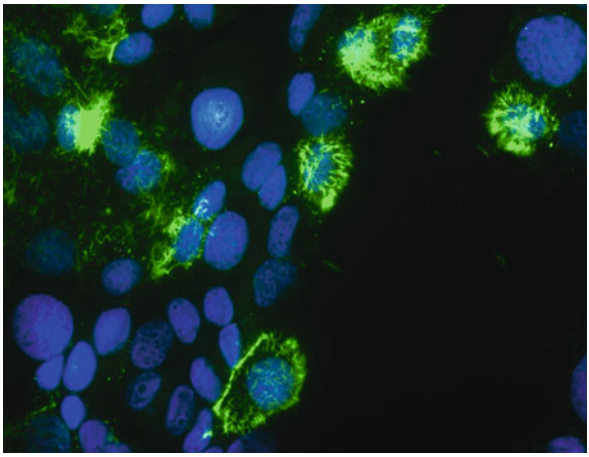

Fig. 5.4 Fluorescent staining pattern of LLC-MK2 cell infected with HMPV (02-001), stained with DAPI and mouse monoclonal antibody to the HMPV N protein (photo courtesy of Dr. F.M. Preston)

5.5.3 Criteria for Test Selection

The laboratory's primary role will most often dictate their method of choice for respiratory virus detection. Diagnostic laboratories require rapid result turn-around and will most likely opt for molecular detection which can be readily adapted to high throughput batching and multiplexing. Most molecular assays can be reported within 24 h of sample receipt whereas culture methods will take from 1 to 14 days and often much longer for confirmation.

Direct antigen tests are available commercially for some respiratory viruses but lack sensitivity (approximately 70%) although the positive predictive value is high. The expense and false negative rate of direct antigen tests need to be balanced with their rapidity and ease of use. Negative direct antigen results should be confirmed by a more sensitive assay such as PCR.

Public health laboratories will most likely employ viral culture to enable viral isolate characterisation and the ability to detect unsuspected viruses.

5.5.4 Commercial Assays

Despite the popularity of molecular detection methods for respiratory viruses very few well validated commercial assays are available for the wide range of respiratory pathogens that are of clinical significance. Most laboratories utilise "in-house" methods in combination with a rigorous quality assurance programmes.

5.6 PCR Detection of Respiratory Viruses

Cell culture and direct immunofluorescent assay (DFA) staining using monoclonal antibodies were previously the most commonly used laboratory techniques for detecting respiratory viruses. Although still used widely in the United States, these traditional techniques have gradually been superseded by highly sensitive and rapid reverse transcriptase polymerase chain reaction (RT-PCR) assays, with most laboratories in Australia now using RT-PCR methods. Additional advantages of RT-PCR detection of respiratory viruses include results that are not significantly affected by a loss of viral viability during specimen transport or storage, and that RT-PCR does not require the presence of intact, infected cells within the specimen. The PCR revolution has also been further stimulated through improvements in the technology, including the advent of real-time PCR and multiplex PCR methods, both of which reduce staff hands-on time, decrease result turnaround-times, increase through-put and are more user friendly compared to conventional PCR techniques.

Standard quality control practices should be implemented when testing for respiratory viruses by PCR, including the use of a suitable positive control, negative control, extraction control and inhibition control. *Sequence-related* issues (discussed in more detail in Chapter 8) are also very relevant to respiratory viruses. Consideration needs to be given to the type of probe used when designing real-time PCR methods for respiratory viruses. The main issue is that respiratory viruses, particularly the RNA viruses, show considerable genetic variation. For this reason, it is often difficult to identify a sufficiently large and conserved region to accommodate two hybridisation probes and so the single-probe TaqMan format is more commonly utilised for respiratory virus detection. On the other hand, we have also observed problems using the smaller minor-groove binder (MGB) TaqMan probes. The issue stems from MGB TaqMan probes being more susceptible to single nucleotide polymorphisms than standard TaqMan probes. For example, we have found that some RSV strains provided poor fluorescent signal, barely above the background negative signal, in an RSV MGB assay [42].

References

1. Allander T, Tammi MT, Eriksson M et al (2005) Cloning of a human parvovirus by molecular screening of respiratory tract samples. Proc Natl Acad Sci U S A 102:12891–12896
2. Allander T, Andreasson K, Gupta S et al (2007) Identification of a third human polyomavirus. J Virol 81:4130–4137
3. Allander T, Jartti T, Gupta S et al (2007b) Human bocavirus and acute wheezing in children. Clin Infect Dis 44:904–910
4. Ambrose HE, Clewley JP (2006) Virus discovery by sequence-independent genome amplification. Rev Med Virol 16:365–383
5. Antachopoulos C, Walsh TJ, Roilides E (2007) Fungal infections in primary immunodeficiencies. Eur J Pediatr 166:1099–1117
6. Arden KE, McErlean P, Nissen MD et al (2006) Frequent detection of human rhinoviruses, paramyxoviruses, coronaviruses, and bocavirus during acute respiratory tract infections. J Med Virol 78:1232–1240
7. Bialasiewicz S, Whiley DM, Lambert SB et al (2008) Presence of the newly discovered human polyomaviruses KI and WU in Australian patients with acute respiratory tract infection. J Clin Virol 41:63–68
8. Bialasiewicz S, Whiley DM, Lambert SB et al (2009) Merkel cell polyomavirus in respiratory specimens: a possible route of transmission? Emerg Infect Dis 15:492–494
9. Brotherton J, Wang H, Schaffer A et al (2005) Vaccine preventable diseases and vaccination coverage in Australia, 2003 to 2005. Commun Dis Intell 31 Suppl:S1–S152
10. Cane PA, van den Hoogen BG, Chakrabarti S et al (2003) Human metapneumovirus in a haematopoietic stem cell transplant recipient with fatal lower respiratory tract disease. Bone Marrow Transplant 31:309–310
11. Carrat F, Vergu E, Ferguson NM et al (2008) Time lines of infection and disease in human influenza: a review of volunteer challenge studies. Am J Epidemiol 167:775–785
12. Chandran A, Watt JP, Santosham M (2008) *Haemophilus influenzae* vaccines. In: Plotkin SA, Orenstein WA, Offit PA (eds) Vaccines, 5th edn. Saunders-Elsevier, UK
13. Cutts FT, Zaman SM, Enwere G et al (2005) Efficacy of nine-valent pneumococcal conjugate vaccine against pneumonia and invasive pneumococcal disease in The Gambia: randomised, double-blind, placebo-controlled trial. Lancet 365:1139–1146
14. Falsey AR, Hennessey PA, Formica MA et al (2005) Respiratory syncytial virus infection in elderly and high-risk adults. N Engl J Med 352:1749–1759
15. Foulongne V, Guyon G, Rodiere M (2006) Human metapneumovirus infection in young children hospitalized with respiratory tract disease. Pediatr Infect Dis J 25:354–359
16. Gaynor AM, Nissen MD, Whiley DM et al (2007) Identification of a novel polyomavirus from patients with acute respiratory tract infections. PLoS Pathog 3:595–604
17. Gerberding KM, Eisenhut CC, Engle WA et al (1989) Congenital candida pneumonia and sepsis: a case report and review of the literature. J Perinatol 9:159–161
18. Han LL, Alexander JP, Anderson LJ (1999) Respiratory syncytial virus pneumonia among the elderly: an assessment of disease burden. J Infect Dis 179:25–30.
19. Hope WW (2009) Invasion of the alveolar-capillary barrier by *Aspergillus spp*.: therapeutic and diagnostic implications for immunocompromised patients with invasive pulmonary aspergillosis. Med Mycol 47 Suppl 1:S291–S298
20. Huston SM, Mody CH (2009) Cryptococcosis: an emerging respiratory mycosis. Clin Chest Med 30:253–264
21. Karron RA (2008) Respiratory synsytial virus and parainfluenza virus vaccines. In: Plotkin SA, Orenstein WA, Offit PA (eds) Vaccines, 5th edn. Saunders-Elsevier, UK.
22. Kieny MP, Girard MP (2005) Human vaccine research and development: an over-view. Vaccine 23:5705–5707

23. Ksiazek TG, Erdman D, Goldsmith CS et al (2003) A novel coronavirus associated with severe acute respiratory syndrome. N Engl J Med 348:1953–1966
24. Lau SK, Woo PC, Yip CC et al (2006) Coronavirus HKU1 and other coronavirus infections in Hong Kong. J Clin Microbiol 44:2063–2071
25. McCullers JA (2006) Insights into the interaction between influenza virus and pneumococcus. Clin Microbiol Rev 19:571–582
26. Metlay JP, Fishman NO, Joffe M et al (2006) Impact of pediatric vaccination with pneumococcal conjugate vaccine on the risk of bacteremic pneumococcal pneumonia in adults. Vaccine 24:468–475
27. (2004) Progress in reducing global measles deaths: 1999–2002. Wkly Epidemiol Rec 79:20–21
28. Rudan I, Boschi-Pinto C, Biloglav Z et al (2008) Epidemiology and etiology of childhood pneumonia. Bull World Health Organ 86:408–416
29. Sato M, Hosoya M, Kato K et al (2005) Viral shedding in children with influenza virus infections treated with neuraminidase inhibitors. Pediatr Infect Dis J 24:931–932
30. Sloots TP, Mackay IM, Bialasiewicz S et al (2006a) Human metapneumovirus, Australia, 2001–2004. Emerg Infect Dis 12:1263–1266
31. Sloots TP, McErlean P, Speicher DJ et al (2006b) Evidence of human coronavirus HKU1 and human bocavirus in Australian children. J Clin Virol 35:99–102
32. Talbot TR, Poehling KA, Hartert TV et al (2005) Seasonality of invasive pneumococcal disease: temporal relation to documented influenza and respiratory syncytial viral circulation. Am J Med 118:285–291
33. Talbot HK, Crowe JE J., Edwards KM et al (2009) Coronavirus infection and hospitalizations for acute respiratory illness in young children. J Med Virol 81:853–856
34. van den Hoogen BG, de Jong JC, Groen J et al (2001) A newly discovered human pneumovirus isolated from young children with respiratory tract disease. Nat Med 7:719–724
35. van der Hoek L (2007) Human coronaviruses: what do they cause? Antivir Ther 12:651–658
36. van der Hoek L, Pyrc K, Berkhout B (2006a) Human coronavirus NL63, a new respiratory virus. FEMS Microbiol Rev 30:760–773
37. van der Hoek L, Sure K, Ihorst G et al (2006b) Human coronavirus NL63 infection is associated with croup. Adv Exp Med Biol 581:485–491
38. Viboud C, Alonso WJ, Simonsen L (2006) Influenza in tropical regions. PLoS Med 3:e89
39. Walter ND, Grant GB, Bandy U et al (2008) Community outbreak of *Mycoplasma pneumoniae* infection: school-based cluster of neurologic disease associated with household transmission of respiratory illness. J Infect Dis 198:1365–1374
40. Weber MW, Mulholland EK, Greenwood BM (1998) Respiratory syncytial virus infection in tropical and developing countries. Trop Med Int Health 3:268–280
41. Weinberg GA, Hall CB, Iwane MK et al (2009) Parainfluenza virus infection of young children: estimates of the population-based burden of hospitalization. J Pediatr 154:694–699
42. Whiley DM, Sloots TP (2006) Sequence variation can affect the performance of minor groove binder TaqMan probes in viral diagnostic assays. J Clin Virol 35:81–83
43. Williams BG, Gouws E, Boschi-Pinto C et al (2002)Estimates of world-wide distribution of child deaths from acute respiratory infections. Lancet Infect Dis 2:25–32
44. Woo PC, Lau SK, Chu CM et al (2005) Characterization and complete genome sequence of a novel coronavirus, coronavirus HKU1, from patients with pneumonia. J Virol 79:884–895
45. World Health Organization (2005) Make any mother and child count. Geneva.
46. Wu B, Liu H, Huang J et al (2009) Pulmonary cryptococcosis in non-AIDS patients. Clin Invest Med 32:E70–E77
47. Zilberberg MD, Shorr AF (2009) Fungal infections in the ICU. Infect Dis Clin North Am 23:625–642