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CHAPTER

# 11

# Molecular Testing for Respiratory Viruses

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## INTRODUCTION

Respiratory tract infections (RTIs) are common and are associated with significant health burden. For example, pneumonia is the fourth leading cause of death globally and the leading infectious cause [1]. Despite being generally mild and self-limiting, the common cold is associated with an enormous economic burden, both in lost productivity and in expenditures for treatment [2]. The major viral agents of RTIs include influenza viruses A and B, respiratory syncytial virus (RSV), human metapneumovirus (HMPV), parainfluenza virus (PIV), adenovirus (AdV), rhinoviruses (RVs), enteroviruses (EVs), and human coronavirus (HCoV). Common to these viruses are their ability to infect airway epithelial cells, co-opt host cell proteins to facilitate infection, modulate both innate and adaptive immune responses, and to mediate proinflammatory responses which contribute to disease pathogenesis (Table 11.1). Yet, some of the unique features of these viruses can lead to diagnostic limitations.

## MOLECULAR TARGETS

### Influenza

Influenza viruses are some of the most important human pathogens, infecting hundreds of millions of people annually with 250,000−500,000 deaths worldwide [3]. As members of the *Orthomyxoviridae* family, these viruses are classified into three distinct types, A, B, and C viruses based on major antigenic differences, subdivisions based on antigenic characterization of the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). Currently, among the type A viruses, there are 16 HA subtypes and 9 NA subtypes.

Influenza infections are usually acute, self-limited, febrile illness which manifest clinically as fever, malaise, and cough with attack rates as high as 10−40% [4]. Their occurrence is generally seasonal with outbreaks of varying severity observed almost every winter. Pandemics have occurred in 1918, 1957, 1968, and 2009 and were caused by different antigenic subtypes of influenza A: H1N1, H2N2, H3N2, and again H1N1 (Fig. 11.1).

Historically, H3N2 is associated with higher mortality [4]. Alternately, other stains are associated with more severe infection among individuals with certain high-risk factors such as obesity, pregnancy, and other comorbidities [5,6]. Furthermore, specific viral mutations are associated with higher virulence and cell receptor binding, which affects their predilection for the upper (URTI) or lower respiratory tract infection (LRTI). The Glu222Gly substitution in the *HA* gene can be found in strains of avian influenza, and to a lesser extent, in some strains of 2009 H1N1 [7,8]. Whereas most strains of influenza replicate in the URT where α-2,6-linked sialic acid receptors predominate on cell surfaces, this amino acid substitution is associated with a greater affinity for α-2,3-linked receptors which are more abundant in the LRT, resulting in a greater risk for viral pneumonia [9−12]. Despite the greater number of influenza A hospitalizations, there appears to be no significant difference between influenza A and B in rates of high-risk conditions, median length of stay, intensive care unit (ICU) admissions, or deaths [13].

Human infection with zoonotic strains is more concerning as these strains have the potential to be more pathogenic, as seen with the avian H5N1 strains, and

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TABLE 11.1 Natural History, Pathogenesis, and Clinical Presentation of Common Respiratory Viruses

| | Seasonality in temperate climates | Incubation period | Duration of illness | Period of shedding[a] | Replication site | Cell receptor | Mechanism of pathogenesis | Presentation | Respiratory disease syndrome(s) | Extrapulmonary manifestation | Optimal specimen |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Flu | Sharp annual peak lasting ~6 to 8 weeks | 1–4 days (average = 2 days) | 3–7 days Cough, malaise more than 2 weeks | Day −1 to day 10 | 1° ciliated columnar, also alveolar and dendritic | Predominately α-2,6-linked sialic acids | H5N1 and to a lesser extent 2009 H1N1, predominately LRT where α-2,3-linked sialic acids predominate on cuboidal bronchiolar cells | Abrupt onset of flu symptoms (fever, myalgia, headache, malaise, dry cough, pharyngitis, and rhinitis). Otitis media, nausea, and vomiting may also be observed | Flu<br>Pneumonia | Cytokine related encephalitis | Usually NP aspirate, washes, and swabs Influenza pneumonia: include BAL, sputum, or throat |
| RSV | RSV broad peak 15–20 weeks (Oct–May) | 3–8 days (average = 5 days) | 7–14 days | Day −3 to day 14 | 1° ciliated columnar, also alveolar and dendritic | Heparan sulfate and nucleolin | Accessory proteins interfere with IFN pathways. Rapid inhibition of Na + transport, resulting in apical fluid accumulation | Begins with rhinorrhea, pharyngitis, cough, headache, fatigue, and fever. Bronchiolitis ± | URI with or without LRI<br>Bronchiolitis<br>Pneumonia<br>Tracheobronchitis<br>Croup | Rare encephalitis, myocarditis | NP aspirate, washes, swabs |
| HMPV | Late winter and early spring biennial pattern | 4–6 days | 5–10 days | 1–2 weeks | 1° ciliated columnar, also alveolar | Heparan sulfate | Accessory proteins interfere with IFN pathways. Rapid inhibition of Na + transport, resulting in apical fluid accumulation | Begins with rhinorrhea, pharyngitis, cough, headache, fatigue, and fever. Bronchiolitis | URI with or without LRI<br>Bronchiolitis<br>Pneumonia<br>Tracheobronchitis<br>Croup | Rare encephalitis | NP aspirate, washes, swabs |
| PIV | PIV1: biennial autumn; PIV2: also autumn; PIV3: endemic with spring time | 2–7 days | 7–10 days | Day −3 to day 20 | Ciliated columnar | PIV1: sialic acids with terminal NeuAcα2-3Gal PIV3: sialic acids with terminal NeuAcα2-6Gal or NeuGcα2-3Gal | Accessory proteins interfere with IFN pathways. Rapid inhibition of Na + transport, resulting in apical fluid accumulation | Begins with rhinitis, pharyngitis, cough (croupy), and hoarseness, usually with fever<br>PIV1 and PIV2: Croup<br>PIV3: Bronchiolitis | PIV1 and PIV2:<br>URI with or without LRI<br>Croup<br>Pneumonia<br>Bronchiolitis<br>PIV3:<br>URI with or without LRI<br>Bronchiolitis<br>Pneumonia<br>Croup | Rare meningitis, hepatic infection | NP aspirate, washes, swabs |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| AdV | Endemic with winter or early spring epidemics | 2–14 days | 3–14 days | 3–6 weeks, some months to years | Nonciliated epithelial persistence within lymphocytes | A, C, E, and F: CAR<br>B and D: CD46 | Destruction of respiratory epithelial cells<br>Disruption of the integrity of cell–cell contact | Fever, pharyngitis, exudative tonsillitis, and cough with or without diarrhea, vomiting and abdominal pain and/or with or without conjunctivitis | URI with or without GI<br>Conjunctivitis<br>Pneumonia<br>Croup<br>Bronchiolitis | Conjunctivitis, GI, cystitis, rare meningitis, myocarditis, myositis | Affected sites: NP aspirate, washes, swabs, throat, BAL, urine stool, blood |
| RV | Endemic with peaks in fall (Aug–Sep) and spring (Apr–May) | 1–7 days (average = 2 days) | 10–14 days (average = 10 days) | Day −1 to day 14 some 3 weeks | 1° ciliated epithelial, also nonciliated | RV A and B: ICAM-1 and LDLR<br>RV C: other but unknown | Nonspecific host inflammatory responses | Rhinorrhea, pharyngitis, cough, headache, malaise, mild fever | URI<br>Asthma exacerbation<br>Bronchiolitis<br>Pneumonia | Rare meningitis, myocarditis | NP aspirate, washes, swabs, tracheal or bronchial aspirate, BAL |
| | | | | | | RV C: other but unknown | | | | | |
| HCoV | Typical HCoV: endemic with peaks in winter to early spring. Peaks in 2–4 years<br>SARS & MERS: zoonotic | Typical HCoV: 2–5 days (average = 3 days)<br>SARS: 4–7 days | Typical HCoV: 3–18 days (average = 7 days)<br>SARS: 7–21 days | Typical HCoV: day 1 to day 21<br>MERS: Day 1 to day 33 | Typical HCoV: ciliated epithelial<br>MERS: alveolar and blood vessel endothelium | 229E: human aminopeptidase N (hAPN);<br>OC43: carcino-embryonic antigen (CEA)<br>NL63: ACE2HKU1: HLA-CSARS: ACE2MERS: dipeptidyl Peptidase IV (DPP4) | Typical HCoV: destruction of upper respiratory epithelial cells<br>SARS: diffuse alveolar damage | Typical HCoV: rhinorrhea, pharyngitis, cough, headache, malaise, mild fever<br>SARS and MERS: fever, cough, dyspnea, malaise, headache. Diarrhea in some | Typical HCoV:<br>URI<br>Pneumonia<br>Bronchiolitis<br>SARS and MERS:<br>Pneumonia<br>GI<br>Renal failure (MERS) | Typical HCoV: None<br>SARS: GI, kidney, liver | Typical HCoV:<br>SARS and MERS: NP and throat, BAL, sputum serum, stool |

[a]*Shedding in children is generally longer than adults. Immunocompromised can shed virus for weeks to months.*

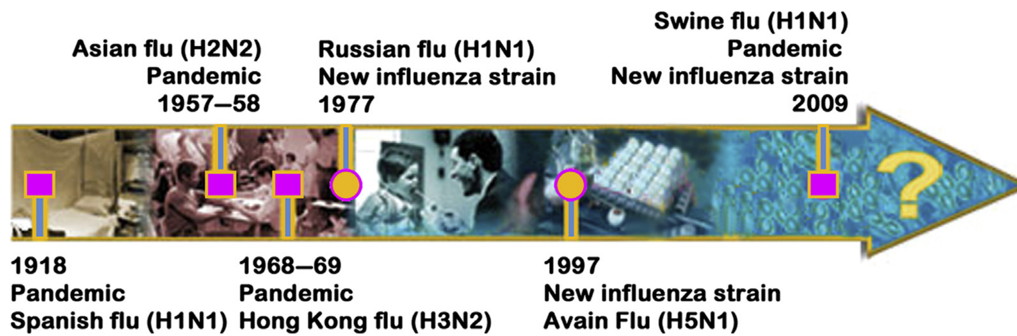


FIGURE 11.1 Timeline of human flu pandemics. Major pandemic; The appearance of influenza strain in the human population. *Source: Adapted from http://www.niaid.nih.gov/topics/Flu/Research/Pandemic/Pages/TimelineHumanPandemics.aspx.*

they have the potential to be the source of the next pandemic due to low levels of immunity in the population. Human infection with many of these strains is associated with unique presentations, such as conjunctivitis with H7 stains, and atypical symptoms like nausea, vomiting, encephalopathy, and bleeding gums and nose with H5 strains [4], which may delay clinical diagnosis and recognition of zoonotic transmission. Interesting, single amino acid changes appear to be responsible for changes in host range [14].

Typically, influenza infections present with systemic symptoms, fever and myalgia, along with upper airway symptoms, such as pharyngitis and dry cough. They usually begin with an abrupt onset of symptoms after an incubation period of 1–2 days and last 4–5 days. However, prolonged infection with or without disease has been reported to last weeks to months in immunocompromised individuals. Less commonly, the virus infects the lung, either via contiguous spread from the URT or via inhalation, causing primary viral pneumonia. Influenza pneumonia frequently requires ICU admission and mortality is high [4]. Secondary bacterial pneumonia is a well-recognized complication of viral pneumonia and accounts for a large proportion of the morbidity and mortality of viral LRT disease, especially in adults. Bronchiolitis and croup may also occur with influenza infection, albeit much less frequently than RSV and PIV. Influenza can be associated with exacerbation of chronic pulmonary diseases such as chronic bronchitis, asthma and worsening pulmonary function in children with cystic fibrosis.

Nonpulmonary complications include myocarditis and pericarditis, as well as exacerbations of other underlying disease such as chronic heart failure and chronic renal disease [15]. Myocarditis is not highly uncommon during influenza infection and may present as asymptomatic myocardial involvement to fulminant myocarditis resulting in cardiogenic shock and death [16]. Central nervous system involvements include the rare occurrence of transverse myelitis and encephalitis which appear to be immune rather than viral mediated [17]. Guillain-Barré syndrome is also associated with immune mechanisms following influenza infection [18].

### *Paramyxoviruses*

RSV and HMPV are from the *Pneumovirinae* subfamily of the *Paramyxoviridae* family. RSV is the major cause of LRT illness in young children and is associated with an estimated 132,000–172,000 pediatric hospitalizations in the United States annually [19] and globally it is an important cause of death [20]. Most infants (50–69%) are infected during the first year of life and virtually all are infected by age 2 [21]. HMPV also causes a broad range of URTI/LRTI, which are clinically indistinguishable from RSV. It accounts for about 1–5% of childhood URTI and 10–15% of hospitalizations for LRTI, depending on age group and year of study [22–24]. Primary infection with HMPV tends to occur at a slightly older age than RSV and by age 5 most children have been infected [25,26].

PIVs also belong to the *Paramyxoviridae* family and are classified as four types and two subtypes (PIV1, 2, 3, 4a, and 4b). PIV1 and to a lesser extent PIV2 are the most significant cause of croup while PIV3 is a significant cause of bronchiolitis, bronchitis, and pneumonia. Indeed these viruses accounted for 6–8% of all hospitalizations for fever or acute respiratory illnesses in children less than 5 years of age [27]. By 5 years of age most children have antibodies against PIV3 and approximately 75% have antibodies against PIV1 and PIV2.

Primary infections with paramyxoviruses are usually symptomatic and present as URTI beginning 2–8 days after infection through the nose or eyes. Although all these viruses replicate in the ciliated columnar cells of the nasopharyngeal (NP) tract [28–30], it is believed that varying cell receptor usage,

including sialic acid containing molecules usage by different PIV strains, likely contributes to the differences in pathogenesis [31,32]. The viruses may then spread to the LRT within 1−3 days as the result of viral impairment of the ciliary epithelium [33]. Paramyxovirus pathogenesis is then associated with necrosis and sloughing of the ciliated epithelial cells which along with edema and increased mucus secretion, obstructs airway, and leads to airway hyperresponsiveness [34,35].

LRTI with RSV and HMPV occurs in 25−40% of cases and manifests most commonly as bronchiolitis, followed by pneumonia and tracheobronchitis, and lastly croup [21,26,36]. Risk factors for bronchiolitis requiring hospitalization include young age, prematurity, male sex for RSV and female sex for HMPV, chronic illness, lower socioeconomic status, smoke exposure, and asthma [21,37−39]. PIV develops into LRTI in 15−25% of cases [40]. There is a tendency for PIV1 and PIV2 to involve the larynx and upper trachea, resulting in the croup syndrome, while PIV3 spreads to the small air passages with the development of bronchopneumonia, bronchiolitis, and/or bronchitis when it is associated with severe disease [27]. There is compelling evidence that the level of virus replication correlates to the disease severity, but innate immune responses also appear to be important [41−43]. HMPV infection appears somewhat milder than that of RSV, but dual HMPV and RSV infections have been reported as more severe than with either virus alone [44,45]. Among the two antigenic subgroups, RSV A is associated with more severe disease than subgroup B [46,47], while the severity of illness associated with HMPV A is similar to HMPV B infection [48].

Reinfection with paramyxoviruses occurs throughout life and is usually present as mild URTI in children and adults with RSV, HMPV, and PIV causing about 7%, 2%, and 5% of acute respiratory illnesses in adults, respectively [49,50]. Reinfection in immunocompromised individuals has a higher risk of more serious disease. Extrapulmonary manifestations from paramyxoviruses are rare and controversial. However, there have been a few reports of paramyxoviruses in CSF in cases of encephalitis or meningitis, as well as in myocardium and liver [43,51−53].

### Adenovirus

Human AdVs, belonging to the genera *Mastadenovirus*, are further divided into seven species (A through G) and 57 types [54]. These viruses cause a broad range of clinical syndromes, with groups A, B, C, and E causing 5−10% of pediatric and 1−7% of adult URTI and LRTI [55]. Several group B AdVs, including serotypes 3, 7, 14, and 21, have caused outbreaks of acute respiratory disease (ARD). Although fatal AdV infections in immunocompetent adults are rare, ARD outbreaks due to a virulent strain of serotype 14 in 2006 and 2007 was associated with a significant number of ICU admissions and deaths in previously healthy young adults [56].

Approximately 50% of all AdV infections result in subclinical disease, and most symptomatic infections are mild and self-resolving within 2 weeks [57]. AdV infection begins with replication in nonciliated respiratory epithelium of the tonsils and adenoids [54]. A brief period of viremia ensues. URTI symptoms in children and young adults include fever, pharyngitis, tonsillitis, and cough, with or without GI symptoms or conjunctivitis [55]. Disruption of the integrity of cell−cell contact enables infection of other cells of the respiratory tract [54]. Worldwide, pneumonia occurs in up to 20% of young children with fatality rates for severe AdV pneumonia exceeding 50% [55]. AdVs utilize cell receptors that are abundantly expressed in epithelial cells in multiple organs or tissues (CAR for groups A, C, E, and F, and CD46 for groups B and D) [58,59]. Hence, extrapulmonary manifestations are common in normal host and include conjunctivitis, GI illness, and cystitis, as well as the more rare occurrences of meningitis, myocarditis, and myositis. AdV can persist as a latent infection for years after an acute initial infection and may reside in lymphoid tissue, renal parenchyma, or other tissues [55]. Reactivation may occur in severely immunosuppressed patients.

AdV causes considerable destruction of respiratory epithelial cells due to inhibition of cellular DNA, mRNA, and protein synthesis resulting in the formation of characteristic smudge cells with enlarged nuclei containing basophilic inclusion bodies surrounded by thin rims of cytoplasm [54]. The penton base structural protein, which causes cells to detachment in vitro, may be involved in pathogenesis in vivo.

### EVs—Including RVs and Human Parechoviruses

EVs and human parechoviruses (HPeVs) of the *Picornaviridae* family are associated with RTI in addition to a wide array of other disease. In fact, EVs are responsible for up to approximately 19% of LRTI in hospitalized children [60]. Human infections are associated with four species of EV (EV A−D), three species of RVs (RV A−C) from the EV genus and one species from the HPeV genus (HPeV A). Although strains from all species may infect the respiratory tract, EV C (C104, C109, C117), EV D (D68), RV A, and RV C are associated with more serious respiratory disease.

RV is undoubtedly the most commonly detected respiratory virus in all age groups, accounting for 25% of all respiratory infections, with asymptomatic infection occurring in at least 20% of healthy individuals

[61]. RV preferentially infects the URT, primarily the paranasal sinuses and nasopharynx. One to three days after infection, URTI frequently begins as a sore or scratchy throat followed by nasal obstruction and rhinorrhea with cough, headache, malaise, and sometimes fever. Large- and medium-sized airways also maintain high-level RV replication [62]. As a result, RV is associated with bronchiolitis in infants and exacerbations in patients with chronic asthma. LRTI such as pneumonia, croup, and bronchitis also occur and result in a significant number of hospitalizations [63]. Cytopathogenicity of this virus is low and pathology is primarily due to nonspecific host inflammatory responses.

### *Coronaviruses*

Most members of *Coronaviridae* family infecting humans (229E and OC43 from the alpha-CoV genus, NL63 and HKU1 from the beta-CoV genus) cause mild URT diseases. In fact, these typical HCoV cause up to 30% of all URTIs [64]. However, two novel beta-CoV, severe acute respiratory syndrome associated CoV (SARS-CoV), and Middle East respiratory syndrome CoV (MERS-CoV) cause serious viral pneumonitis, leading to hospitalization and death with overall mortality rates of 10% and 30%, respectively [65].

Infection with the typical HCoV, of which 30% are asymptomatic, begins with replication in the ciliated epithelial cells of the nasopharynx. Direct destruction of ciliated epithelial cells in conjunction with innate immune responses produces rhinorrhea, pharyngitis, cough, headache, malaise, and mild fever 2–5 days after infection. These viruses have also been associated with severe pneumonia and bronchiolitis in neonates and the elderly, especially those with underlying illnesses. In addition, HCoV-NL63 is also an important cause of croup [66]. Infection frequently occurs in young children with seropositivity in 50% of school-age children [64]. Reinfection as well as coinfection is common.

SARS begins with fever, headache, malaise, or myalgia, followed by nonproductive cough and dyspnea in a few days to a week after onset of symptoms. Although the upper airway is also infected, there is little epithelial cell damage and URT disease is lacking. Virus rapidly spreads to the alveoli, causing diffuse alveolar damage leading to pneumonia and ARDS in 25% of cases [67]. Diarrhea is common. MERS is also associated with a biphasic illness strikingly similar to SARS except for more frequent renal failure [68]. Most patients who are hospitalized with SARS and MERS have chronic comorbidities. Interestingly, asymptomatic infections with both viruses have been reported [69,70].

## CLINICAL UTILITY OF MOLECULAR DIAGNOSTICS FOR RESPIRATORY VIRUS INFECTION

Respiratory viruses can infect both the URT and the LRT (Fig. 11.2) and tend to cause distinct clinical syndromes based on their tropism for different sites of the respiratory tract. Most commonly these viruses only

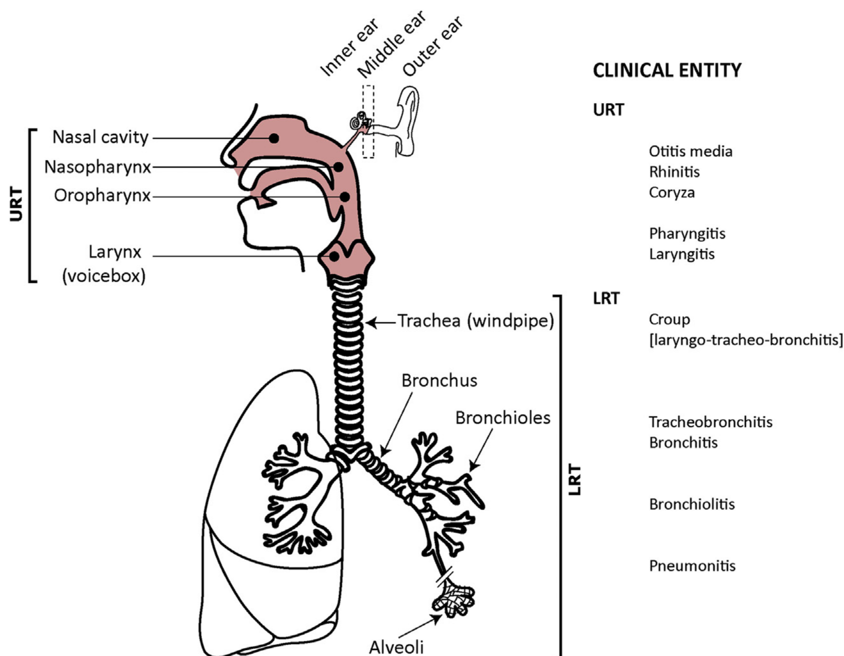


FIGURE 11.2 Schematic representation of the human respiratory tract. The upper (shaded pink) and lower respiratory tract (URT/LRT) and the components of the ear are indicated. The approximate locations of URT and LRT diseases associated with respiratory virus infection are indicated. *Source: Reprinted with permission from Caister Academic Press (from Mackay IM, Arden KE, Nissen MD, Sloots TP. Challenges facing real-time PCR characterization of acute respiratory tract infections. In: Mackay IM, editor. Real-time PCR in microbiology: from diagnosis to characterization. Caister Academic Press; 2007. pp. 269–318).*

infect the URT, and when LRT infection does occur, it is most often due to contiguous spread.

## Upper Respiratory Tract Infections

### *The Common Cold*

The common cold refers to a syndrome of upper respiratory symptoms that may be caused by a variety of viral pathogens. These symptoms include nasal blockage, runny nose, sneezing, cough, and sore throat, sometimes with headache or other body aches, and typically begin 1–3 days after infection. Fever and other constitutional symptoms are more often seen in URTIs associated with influenza, RSV, HMPV, and AdV. Colds usually last about 1 week, but virus shedding can persist for 2–3 weeks. Otitis media can develop from URTI with any of these viruses and can due to secondary bacterial infection or direct viral infection. Indeed, virus can be detected in middle ear fluids with RSV, influenza, HCoV, and RV being the most common [71].

The pathogens most frequently associated with common cold symptoms are the EV/RV, which cause approximately half of all colds in children and almost three-quarters of colds in adults, and HCoV (Table 11.2). It is often forgotten that influenza viruses can present with only mild URTI symptoms and is in fact a common cause of the cold. Other important pathogens that are also associated with cold symptoms include AdV, RSV, HMPV, and PIV. Coinfections are common.

Although generally mild and self-limited, these illnesses are associated with an enormous economic burden both in lost productivity and in expenditures for treatment. Hence, attempts have been made to create and market antiviral agents targeting causes of the common cold, particularly EV/RV [72]. Due to the lack of success in therapeutic interventions, diagnostic testing outside of epidemiological investigations is not warranted.

### *Influenza-Like Illness*

Influenza-like illness (ILI) is on the other end of the spectrum of URTIs and is defined as the presence of fever of greater than or equal to 100°F, in addition to cough or sore throat, in the absence of an alternative cause. After an incubation period of 1–4 days, there is an abrupt onset of constitutional and respiratory signs and symptoms which generally lasts 5–7 days. The constitutional symptoms can include malaise, body aches, headache, loss of appetite, and nausea and are generally due to cytokines released by immune system activation. Interestingly, influenza only causes 35–45% of ILI cases during peak seasons. But many other viral infections can present as flu-like, particularly RV/EV and RSV (Table 11.2).

Appropriate treatment of patients with respiratory illness depends on accurate and timely diagnosis. Early diagnosis of influenza can reduce the inappropriate use of antibiotics, provide the option of using antiviral therapy and is an important infection prevention measure. The causative agent of ILI is difficult to determine on the basis of signs and symptoms alone. Sensitivity and predictive value of clinical definitions vary, depending on the prevalence of other respiratory pathogens and the level of influenza activity. Among generally healthy adults living in areas during the peak of influenza activity, the positive predictive value (PPV) of a simple clinical definition of influenza (acute onset of cough and fever) can be over 80%. However, the presentation in children, the elderly, and individuals with comorbidities is less likely to be typical, in

**TABLE 11.2** Relative Rates of Respiratory Viruses Among Respiratory Tract Syndromes

| Virus | Common cold (pediatric/adult) | ILI[a] (all ages) | Croup (pediatric) | Bronchiolitis (pediatric) | Pneumonia (pediatric/adult) |
|---|---|---|---|---|---|
| AdV | 5–10/1 | 0.4–9 | 1 | 1–8 | 1–10/3–13 |
| HCoV | 10–15/11 | 0.2–10 | 2[b] | 1–8 | 3–7/6–13 |
| Influenza | 25–30/8 | 8–52 | 9 | 1–10 | 4–22/21–31 |
| HMPV | 1–5/1 | 0.2–10 | <1 | 3–12 | 1–13/3–22 |
| PIV | 1–5/5 | 0.4–11 | 42[c] | 1–3 | 8–28/6–14 |
| RSV | 1–5/3 | 0.4–19 | 15 | 70–80 | 3–45/13–24 |
| RV/EV | 40–50/71 | 4–29 | 21 | 15–35 | 3–45/13–24 |
| References | [74,124] | [79,138,139] | [140] | [141–143] | [77,144] |

[a]*Influenza-like Illness = fever, myalgia, pharyngitis, and dry cough.*
[b]*Frequencies not yet well established.*
[c]*PIV1 = 31%, PIV2 = 5%, PIV3 = 6%.*

which case the PPV of clinical impression can be as low as 17−30% in these populations [73].

Dagnostic testing is not needed for all patients with ILI to make antiviral treatment decisions once high levels of influenza activity have been identified in the region. For most outpatient and emergency room settings, results for molecular assays are generally not available to assist in clinical decision making. Fortunately, that paradigm is changing with advent of rapid tests which provide a wide panel of results in approximately 1 h, or 20-min point-of-care devices for influenza. But generally, molecular testing has been considered to be most appropriate for hospitalized patients if a positive test would result in a change in clinical management, including infection control practices.

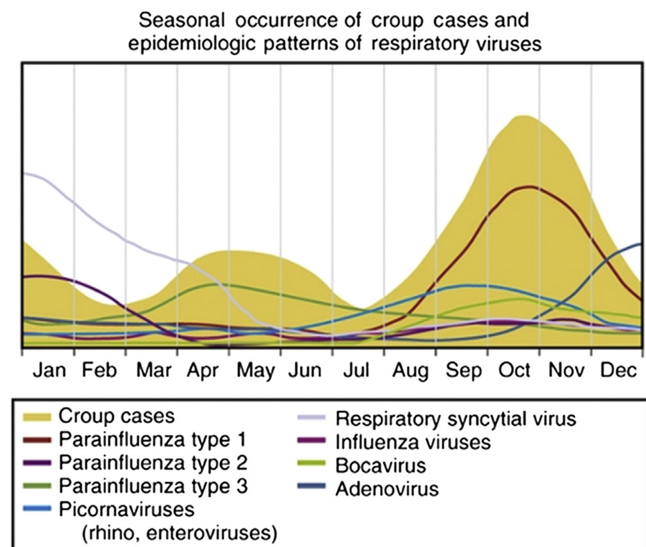


FIGURE 11.3 The seasonal occurrence of croup cases is shown in relation to the epidemiologic activity of the respiratory viruses associated with croup. *Source: Reprinted from Hall and McBride (Bower J, McBride JT. Croup in children (acute laryngotracheobronchitis). In: Bennett JE, Dolin R, and Blaser MJ, Editors. Mandell, Douglas, and Bennett's principles and practice of infectious diseases, 8th ed. Philadelphia, PA: Saunders/Elsevier; 2015, pp. 762−766).*

## Lower Respiratory Tract Infections

### *Croup*

Croup is a common childhood disease characterized by sudden onset of a distinctive barky cough that is usually accompanied by inhalation stridor, hoarse voice, and respiratory distress resulting from upper airway obstruction that worsens at night. Although the illness is generally mild and short-lived, the presentation in a child is alarming. In fact 85% of cases typically present mild croup and fewer than 5% are hospitalized [74].

Typically, this disease affects children between 3 months and 3 years of age. Frequently it begins with a nonspecific URTI 12−48 h prior to the development of classic symptoms. The barky cough resolves within 3−4 days for 60% of cases, but some patients will continue to have symptoms for up to 1 week [74].

Although present year-round, croup often presents with biannual peaks in late autumn and again in spring, particularly in odd-numbered years, correlating with the prevalence of PIV (Fig. 11.3). Of the PIV strains, type 1 is the primary cause of croup, followed by type 3, and then 2 [74]. This finding appears contradictory since type 3 is usually associated with bronchiolitis. However, this observation is easily explained by the greater prevalence of type 3 virus over type 2 virus. Other viruses implicated in the disorder include influenza, AdV, RSV, HMPV, and HCoV-NL63. In addition, measles remains an important cause of croup in nonimmunized children. RV coinfection is frequent.

Croup is a clinical diagnosis. Laboratory tests are not needed to confirm the diagnosis. Laboratory analysis generally should be limited to tests necessary for management of a more severely ill child. Viral identification may be warranted when specific antiviral therapy is being considered, such as for severely ill or high-risk children with influenza.

### *Bronchiolitis*

Bronchiolitis is the most common acute viral LRT illness in children less than 2 years of age. Clinical signs and symptoms of bronchiolitis include rhinorrhea, cough, tachypnea, wheezing, rales, and increased respiratory effort which typically lasts 3−7 days. There is commonly a prodromal URTI (coryza, cough, and mild fever) which lasts for several days. Complications with bronchiolitis, such as apnea and aspiration, occur most frequently in infants within the first several months of life, in premature infants, and in children with chronic conditions. Indeed, it is the most common cause of hospitalization among infants during the first 12 months of life. Although the hospitalization rates for bronchiolitis have been increasing, mortality rates have declined [75].

Peak occurrence of bronchiolitis is during the winter to early spring, and usually correlates with the prevalence of RSV, which causes of about 70−80% of cases. RV/EV and HMPV are other leading cause of bronchiolitis, but all respiratory viruses have been associated with bronchiolitis (Table 11.2), and a considerable fraction of cases (30%) involve multiviral infections. Again, bronchiolitis is a clinical diagnosis and laboratory tests are not needed to confirm the diagnosis. In fact, the American Academy of Pediatrics recommends against radiographic or laboratory studies routinely [76].

### *Pneumonia*

Pneumonia is a common illness with high morbidity and mortality, particularly in children less than 5 years old and in adults over 75. Viruses are more commonly associated with pneumonia in children, particularly influenza, RSV, RV, HMPV, and PIV (Table 11.2) [77]. The prevalence of the causative agents is age-dependent, with RSV and PIV being more common causes of pneumonia in children less than 2 years old than in older children. Dual viral infections are common, and a third of children have evidence of viral−bacterial coinfection, particularly with *Streptococcus pneumoniae* and *Staphylococcus aureus*. In adults, viral agents are an important cause of pneumonia in the elderly, although historically their role has been underestimated given the insensitivity of antigen assays and viral culture in this population. As the result of nucleic acid amplification testing, it is now evident that viruses, in particular influenza viruses, RVs, and coronaviruses, are the putative causative agents in a third of cases of community-acquired pneumonia [78,79].

Viral infection of the lung can be the result of either contiguous spread from the URT or by direct inhalation, with the former beginning with typical URTI symptoms followed by a rapid progression of fever, cough, dyspnea, and cyanosis. Nonrespiratory symptoms include fatigue, sweats, headache, nausea, and myalgia. With increasing age, both respiratory and nonrespiratory symptoms of pneumonia become less frequent. Primary viral pneumonia frequently requires ICU admission and mortality is high. The diagnosis of pneumonia is determined clinically and confirmed by radiographic imaging, but identification of the etiological agent is important and recommended by the Infectious Diseases Society of America and the American Thoracic Society [80]. Indeed, viral pneumonia cannot be differentiated from bacterial pneumonia clinically, particularly in the elderly. Furthermore, secondary bacterial infection with certain bacteria may be virus-specific, increasing the need to know the causative agent [81].

## MOLECULAR TECHNOLOGIES AND LIMITATIONS OF TESTING

Since most cases of viral respiratory infection (VRI) are associated with mild, self-limiting illness, laboratory testing is not necessary. However, for more serious cases, such as those requiring hospitalization or therapy, rapid laboratory diagnosis of the etiological agent can be important. Viral diagnostics can guide therapy, potentially eliminating unnecessary use of antibiotics and enabling the use of antivirals when appropriate. In addition, knowledge of the causative agent is important for infection control interventions to minimize the risk of nosocomial spread. Nucleic acid amplification tests (NAATs) have become the test of choice for VRI because rapid antigen tests have low sensitivities [82−86] and viral culture, which can take 3−10 days, lacks utility for patient management. Furthermore, NAATs have superior sensitivity and specificity in both pediatrics and adults, and results can be obtained within minutes to hours [87−89]. Not only has NAAT revolutionize the detection of traditional respiratory viruses with its exquisite sensitivity, but it enabled the discovery of new respiratory viruses, such as HMPV, many HCoV, and RV C group.

More recently, multiplexed molecular assays have been developed in order to diagnose a large number of respiratory viruses in single assays. As an added benefit, viruses that could not be detected by conventional virology have been included which further increases the diagnostic yield. Refer to the review by Gaydos [90] for details regarding the performance and workflow of many of these systems. The principle differences among NAATs are the throughput, turnaround time, ease of use, automation, versatility, use of a closed system to reduce contamination and cost.

Early problems with NAAT included lack of sensitivity for specific subtypes of AdV, the inability to differentiate RV from EV, and contamination issues with open platforms [91]. As expected manufacturers made or are working to make improvements, such as enhancing the range of AdV strain detection and reducing to eliminating postamplification processing [92]. In addition, point-of-care tests are now available, some with 20-min turnaround times.

The advantage of NAAT for VRI in terms of cost reduction is still unclear. Whereas rapid antigen tests have been shown to reduce length of stay, performance of ancillary diagnostic tests, and antibiotic consumption, the same cannot be said for multiplex assays, despite their higher sensitivity and specificity, and capacity to detect an extended range of viruses [91,93]. Initially, available multiplex systems were geared toward batched workflow usually performed once or twice per day. Subsequently, on-demand amplification methods, with potential turnaround time of 1−2 h, have come to market and are replacing batched test systems. Small studies have begun to show that identifying viral pathogens within a few hours does impact antibiotic or antiviral use and reduces labor cost in the Emergency Department [94,95]. More studies are needed to see if these results hold true.

### Specimen Collection

The general rule for optimal specimens for the diagnosis of viral infection dictates that the specimens

originate from the site of viral replication. Respiratory viruses are no different and since site of replication for these viruses is primarily the URT, in particular the NP region, it is best to sample that region for diagnostic testing. Of the URT samples, NP aspirates have traditionally been considered the most sensitive specimen for the detection of respiratory viruses [96,97]. However, a recent review by Jartti et al. [98] indicates that at least in children all NP samples, aspirates, washes, swabs, or brushings, have statistically equal sensitivity for NAAT, particularly when flocked swabs are used [99]. Historically, virus recovery in adults is much more difficult than in children because virus titers tend to be much lower in adults. The analytical sensitivity of NAAT appears to negate this concern [100]. Similarly, when flocked nasal swabs are used the sensitivity is similar to NP specimens in both pediatric and adult patients [100,101]. In addition, self-collected (in adult patients) or parent-collected flock nasal swabs specimens also show equivalency, opening the door for point-of-care devices [102−104].

Respiratory viruses can also be isolated from throat swabs or washes. Although the viral yield is typically lower than that seen with NP specimens, combining a throat swab and an NP swab may improve virus detection [87,105,106], and the general consensus is that throat swab alone is not recommended for most viruses. Exceptions include AdV, which replicates in the tonsils, and avian influenza, which primarily replicates in the LRT.

Calcium alginate swabs and swabs with wood shafts should not be used for respiratory specimen collection because they may interfere with NAATs. Specimens should be placed in sterile viral transport medium and refrigerated until transported to the laboratory for testing as soon as possible. However, some NAAT assays are approved for room temperature transport provided it occurs within a few hours. Clinicians should be aware of the approved clinical specimens, as well as specimen storage and transport, for the molecular assay being ordered. Freezing and thawing should be avoided or minimized to avoid degradation of virus particles, exposing the viral RNA to nucleases. Also viral integrity is needed if viral culture is to be performed, for example, for influenza resistance testing.

For cases of LRTI, sputum, endotracheal aspirates, or bronchoalveolar lavage specimens can increase the PCR diagnostic yield and should certainly be considered when URT specimens yield negative results but the suspicion is high. This is particularly true for viral pneumonia due to influenza, especially for cases of LRTI due to inhalation rather than spread from the URT. In fact, false-negative NP test occur in 10−35% of patients with viral pneumonia [107,108]. However, LRT specimens are not recommended for routine use as the diagnostic yield will not significantly improve. Furthermore, specificity is not necessarily improved with LRT specimens, particularly with the use of NAAT, as virus can be detected in LRT samples from asymptomatic children [98]. Lastly, none of the commercially-available tests have been validated for use with LRT specimens.

Although some respiratory viruses are shed from other sites such as urine or stool, as seen with AdV, these specimen types are not recommended for the diagnosis of respiratory illness. The only exceptions are SARS- and MERS-CoV where stool specimens may provide additional diagnostic yield. Rare occurrences of extrapulmonary manifestations have been reported with some respiratory viruses. Indeed, there have been anecdotal reports of respiratory virus detected in the CNS, myocardium, liver, and other sites [109]. Most often extrapulmonary syndromes are not due to a direct viral effect, but rather due to cytokine release as seen with influenza-associated encephalitis or myocarditis. In such cases, influenza RNA is almost never detected in CSF or myocardium. Likewise, rare cases of HMPV-associated encephalitis have been reported, but viral RNA has not been detected in CSF [53]. In contrast, AdV replicates in multiple organs and tissues. For example, AdV is often detected in urine, CSF, or myocardium in cases of AdV-associated hemorrhagic cystitis, meningitis or encephalitis, and myocarditis [55,110]. AdV DNA can even be found in serum during respiratory illness [55].

## Timing of Disease/Virus Shedding

Generally peak respiratory virus shedding occurs on the first or second day of acute illness and generally declines substantially after 4 days [111−115]. However, duration varies with virus, patient age, severity of illness, comorbidities, and immune status. Often hospitalized patients, particularly those with LRTI, have higher viral titers and shed virus longer [111−114,116,117]. NAATs detect viral targets for a longer duration than other test methods and it is not unusual to detect viral nucleic acid a couple of weeks after infection, albeit the mean duration is generally 6−14 days [111−114,118,119]. Of the Paramyxoviruses, the duration of shedding for HMPV may be relatively shorter while PIV3 maybe longer [115,120]. Some viruses, particularly AdVs and picornaviruses, exhibit prolonged shedding in both asymptomatic and symptomatic patients, which can be a diagnostic conundrum [119,121].

Prolonged shedding of all respiratory viruses is not uncommon in severely immunocompromised patients and viral nucleic acids have been detected months after infection [109,118,119,122]. Prolonged shedding of influenza can be observed in this population even in the presence of treatment with antiviral agents. This then has been associated with the development of drug resistance mutations and subsequent community spread of resistant strains [118].

The literature has been inconsistent about the correlation between viral loads and disease severity [91,93]. Part of this discrepancy may be due in part to variances in the viruses themselves. More studies are needed to interpret the significance of viral loads. Likewise, there are mixed reports concerning the associations between infections with multiple viruses and more severe disease [91,93]. Indeed, asymptomatic, prolonged shedding associated with AdVs and picornaviruses complicates the interpretation.

## Seasonality

In regions with temperate climates, the seasonal incidence of respiratory viruses is as diverse as the number of species associated with RTI, but the majority of infections occur between fall and early spring. In tropical climates, infections occur year-round or with increased incidence during the rainy season. The seasonal diversity of respiratory viruses is most evident with epidemiologic patterns of respiratory viruses associated with croup (Fig. 11.3) [123]. Influenza tends to produce a sharp annual peak lasting 6–8 weeks, while RSV tends to have a longer duration on the order of 15–20 weeks [109]. HMPV typically appears in late winter through early spring with a biennial pattern of epidemics [24]. Classically, PIV1 causes autumn epidemics in odd-numbered years and is sometimes accompanied by PIV2. PIV3 is more endemic with peaks in spring to early summer. Seasonality of PIV4 has not been as well characterized [109]. AdV infections occur throughout the year, but most epidemics occur in the winter or early spring [55]. EV infections usually occur in late summer to early fall, but those associated with respiratory disease also tend to be associated with sporadic outbreaks which can occur year-round. RV infections also occur throughout the year, but distinct peaks of illness are seen in the fall and spring [124]. HCoV are more endemic, but a bit more common in the cooler months [109]. Factors affecting seasonality in temperate climates are most likely due to environmental factors such as low temperatures and humidity, as well as social factors associated with colder months such as crowding indoors [125,126].

## Interpretation of NAAT Results

Despite the high sensitivities and specificities of NAAT for respiratory virus detection, false-negative results can occur due to improper specimen collection or handling. A negative result can also occur when the patient is no longer shedding detectable virus, or at least at the site of collection. For hospitalized patients with LRT disease, if no other etiology is identified and viral pneumonia is still clinically suspected, the CDC recommends collecting LRT specimens. Sequence deviations or mutations at the site of primer or probe binding are also a potential source of false-negative results. In 2015, the majority of circulating influenza virus in the United States was characterized as A/Switzerland-like H3N2 viruses with significant genetic drift, loss of vaccine protection, and reduced ability to culture in many cell lines. Indeed, matrix gene primer or probe mismatches affect the performance of some commercial NAATs [127]. On the other hand, sequence deviations in the *H1* gene affected typing of A(H1N1)pdm09, leading to the serendipitous discovery of a strain of influenza A that cannot be typed. Similarly, pan-detection NAATs may not adequately detect all subtypes within a family of virus as commonly seen with commercial assays for the detection of AdV [128–130].

False-positive results, although rare, can occur (eg, due to lab contamination or other factors). A positive result indicates detection of viral nucleic acid, confirming virus infection, but does not necessarily mean the virus is the causative agent. Furthermore, patients vaccinated by intranasal administration of live attenuated influenza virus will likely test positive for 7–10 days [131].

## Antiviral Resistance Testing

Antiviral resistance among influenza strains is a public health concern because resistant strains have spread rapidly in the community, quickly becoming the predominant virus [132,133]. Currently, circulating influenza A (H3N2) and 2009 H1N1 viruses are primarily susceptible to oseltamivir and zanamivir, but are resistant to the adamantanes (amantadine and rimantadine) [133]. However, prior to the 2009 pandemic the seasonal H1N1 virus developed into a predominantly oseltamivir-resistant strain [132]. Luckily this virus was susceptible to the adamantanes. Although only sporadic cases of oseltamivir resistance have been observed in isolates of A(H1N1)pdm09, this virus does have the sample potential as the seasonal H1 strain to become universally resistant.

Phenotypic susceptibility testing remains the gold standard for the assessment of viral resistance, but

some of the more common resistance mutations have been identified which are useful for more rapid identification. For example, a histidine to tyrosine amino acid substitution at residue 275 of the NA protein (H274Y in N2 numbering; H275Y in N1 numbering) is associated with oseltamivir resistance [132]. Similarly, a change in amino acid 31 in the M2 gene product is associated with resistance to adamantanes [134]. Other potentially important mutations include a D199E mutation which is associated with reduced susceptibility to oseltamivir in seasonal H1N1 [135], a D198G (universal numbering, equivalent to site 199) mutation in H5N1 is associated with reduced susceptibility to oseltamivir and zanamivir [136], and a D198N mutation in influenza B virus is associated with high oseltamivir resistance [137].

It is important to point out that such testing is not available in most clinical laboratories and is generally performed by some public health laboratories or at the CDC for epidemiological purposes. Detection of the H275Y mutation is usually determined by a pyrosequencing assay developed by the CDC, while Sanger sequencing is used to assess mutations in the *NA* and *M2* genes.

## CONCLUSIONS

Molecular diagnostics has revolutionized our ability to detect RTIs by increasing sensitivity of virus detection, broadening the array of viruses detected, and enabling the detection of multiple infections. Furthermore, test results are available in a timeframe that can better impact patient management. These tools have also advanced our understanding of the epidemiology and pathogenesis of respiratory virus disease.

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