

Chapter 41

Respiratory Pathogens

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Introduction

Respiratory tract infections are among the most common presenting complaints of patients in both hospital and community settings. They are a considerable burden in terms of both patient morbidity and public health interventions. Laboratory diagnosis of respiratory tract infections should provide guidance in therapy and prognosis, as well as useful epidemiological information reflecting trends in the community. Understanding and monitoring such trends facilitates early recognition of new infectious agents in a population. A summary of the common viruses and bacteria causing respiratory tract infections and their clinical relevance is given in Tables 41-1 and 41-2, respectively.

Even with a significant clinical effort and analysis of multiple specimens, current laboratory methods fail to diagnose approximately half of lower respiratory tract infections. In fact, laboratory diagnosis of community-acquired pneumonia (CAP) is so poor that current clinical practice guidelines do not recommend testing for all but the most severely affected patients and advise use of empiric therapy.¹ This pragmatic approach fails to address issues of antimicrobial overuse and resistance, public health surveillance, and advancement of medical knowledge.

Many “atypical” bacteria are known to cause severe respiratory symptoms, but lack of good diagnostic procedures has hampered the measurement of the real impact of such infections in the community. Despite vaccination policies, *Bordetella pertussis* infection remains relatively common in children and adults and is associated with chronic cough in adults.² *Mycoplasma pneumoniae*, *Legionella pneumophila*, and *Chlamydia pneumoniae* (previously *Chlamydia pneumoniae*) are all recognized causes of lower respiratory tract infections, but again, their impact has not been studied in detail. In addition, despite the well-recognized association of viral infections with upper and lower respiratory tract infections, the current diagnostic virology procedures do not provide an answer rapidly enough to

prevent inappropriate antibiotic use or to consider use of antiviral therapy.^{3,4}

Molecular techniques have the potential to enhance our diagnostic approaches to respiratory pathogen identification and enable more detailed analysis of outbreaks. Use of nucleic acid detection methods has demonstrated that some organisms are more common and important causes of respiratory infection and disease than previously appreciated. Molecular methods are applied to the diagnostic detection and analysis of the viral and some of the atypical bacterial causes of respiratory infections.

Clinical Utility

Limitations of Conventional Diagnostic Procedures

Conventional diagnostic techniques (culture, antigen, and antibody detection) have been widely utilized for the diagnosis of individual infections and for the identification of respiratory outbreak pathogens with varying success. Culture-based methods are the mainstay for the diagnosis of more typical bacterial infections, such as *Streptococcus pneumoniae* and *Staphylococcus aureus*, and provide isolates for antimicrobial susceptibility testing. For many agents of CAP, however, culture methods have significant drawbacks. In particular, such diagnostic approaches have very low sensitivity for atypical bacteria, due to the fastidious nature of the organisms (e.g., *M. pneumoniae*, *C. pneumoniae*, *Bordetella pertussis*), and are too slow to influence patient management. In other cases, culture methods are hazardous and require enhanced containment laboratories (e.g., *Chlamydia psittaci*, *Coxiella burnetii*, *Francisella tularensis*, *Yersinia pestis*), which is a costly and not widely available option.

Cell culture for respiratory viruses is cumbersome, expensive, and available only in major medical centers. For many viral infections, suitable culture techniques and antibodies for isolate identification are not available. Thus, infections

Table 41-1. Respiratory Viruses in Acute and Public Health Settings

Viruses	Acute Infection	Public Health Significance and Community Impact	Conventional Diagnostics	Molecular Diagnostics and Typing
Influenza viruses (A and B)	Mild to severe URT and LRT infection	Responsible for outbreaks in the community and in immunocompromised individuals	DFA or culture can be utilized, but sensitivity depends on sample quality	Utilized for subtype and strain identification, particularly when culture is inefficient or impractical
Parainfluenza viruses (HPIV1-4)	Mild to severe URT and LRT infection	Impact of parainfluenza 4 not clear; others associated with outbreaks in the community and in immunocompromised individuals	DFA or culture can be utilized, but sensitivity depends on sample quality; HPIV4 is not usually identified	Some outbreaks investigated by sequencing to confirm relationship among viruses
Coronaviruses (and SARS-CoV)	Thought to be mild for most coronaviruses; SARS-CoV has a high morbidity and mortality	SARS-CoV spread by close contact and responsible for outbreaks in multiple countries; other coronaviruses not well studied	Not readily available and lack sensitivity; antibody responses slow to develop	Used to confirm SARS-CoV as a new introduction to man; sequencing used to differentiate among coronaviruses
Rhinoviruses	Usually mild in healthy individuals; exacerbations in asthmatic individuals reported	Common cause of mild URT symptoms with significant economic impact	Not readily available and lack sensitivity	Of only academic interest to date but potentially useful for studying the impact of this common virus in the community
Enteroviruses	Recognized as a cause of wide-ranging respiratory symptoms	Not well established for respiratory symptoms but recognized for other clinical manifestations	Only a proportion is culturable; DFA not established for all serotypes	Range of methods used to mirror serotyping procedures; molecular methods likely to replace typing methods that depend on culture
Respiratory syncytial virus (RSV)	Cause of mild to severe infection, particularly in infants and elderly	Responsible for outbreaks in the community and in immunocompromised individuals	DFA or culture can be utilized, but sensitivity depends on sample quality	Differentiated into subtypes RSV A and B based on sequence differences, but clinical relevance unproven
Metapneumovirus	Studies to date indicate clinical presentation similar to that of RSV	Common infection; likely responsible for outbreaks in the community and in immunocompromised individuals; common copathogen	Culture thought to be insensitive; DFA not yet available	Differentiated into two lineages based on sequence differences, but clinical relevance unproven
Respiratory adenoviruses	Mild to severe respiratory infection (URT and LRT)	Responsible for outbreaks in the community and in immunocompromised individuals	DFA or culture can be utilized, but sensitivity depends on sample quality	Differentiated into types and subtypes with recognized clinical relevance; persistence may create problems in interpretation
DFA, direct fluorescent antibody; HPIV, human parainfluenza virus; LRT, lower respiratory tract; RSV, respiratory syncytial virus; SARS-CoV, severe acute respiratory syndrome coronavirus; URT, upper respiratory tract.				

Table 41-2. Respiratory Bacteria in Acute and Public Health Settings

Organism	Acute Infection	Public Health Significance and Community Impact	Conventional Diagnostics	Molecular Diagnostics and Typing
<i>Bordetella pertussis</i>	Mild to severe infection, especially in infants	Large community outbreaks are common	Culture and DFA have low sensitivity; latter also compromised by artifacts	PCR superior to culture but requires standardization; gradual evolution of strains reported
<i>Legionella</i> species	Mild to fatal infection	Frequent outbreaks, water or soil related	Culture performs well, but is slow; Urine antigen detection useful for <i>L. pneumophila</i> serogroup 1 in high-prevalence areas	PCR similar to culture for <i>L. pneumophila</i> ; likely better for non- <i>pneumophila</i> species; typing may be useful for outbreaks, but available data are limited
<i>Mycoplasma pneumoniae</i>	Common cause of CAP, usually mild	Endemic with occasional epidemics	IgM test useful for primary infection	PCR useful for rapid, sensitive detection
<i>Chlamydomphila pneumoniae</i>	Common cause of CAP requiring hospitalization	Community outbreaks described	Limited; serology by MIF most accurate, but slow	PCR useful for rapid, sensitive detection; role of <i>C. pneumoniae</i> prolonged infection or colonization needs to be determined

DFA, direct fluorescent antibody; CAP, community acquired pneumonia; MIF, microimmunofluorescence; PCR, polymerase chain reaction.

with parainfluenza virus type 4, human coronaviruses, rhinoviruses, and some enteroviruses would not ordinarily be identified without RNA detection methods. The impact of such infections is only just being realized, and there is probably an underestimation of their clinical importance, particularly for immunocompromised individuals, the elderly, or those with underlying conditions such as asthma.^{5,6}

Human metapneumovirus has been confirmed as an important cause of severe lower respiratory tract infection. The virus has been circulating for more than 50 years, and studies using molecular assays have confirmed its wide distribution,^{7,8} but many laboratories have not been successful in isolating this virus. The recent identification of the agent causing severe acute respiratory syndrome (SARS), known as SARS-CoV, has illustrated the need for expansion of diagnostic testing to encompass new emerging viruses and the limitations of conventional virological laboratory approaches to respiratory pathogen diagnosis.^{9,10}

Even if it is possible to culture viruses efficiently, isolation and confirmation of the cause of a cytopathic effect can take days to weeks, depending on the pathogen. Waiting for a culture-positive result can take many days, during which time the patient may be inappropriately treated and, if hospitalized, infection control measures may not be initiated. Also, the use of primary primate cells in culture (which gives the best yields of influenza and parainfluenza viruses) is unlikely to be sustainable in the long term.

When available, monoclonal antibodies are useful in direct virus-specific antigen detection tests, and these can be used for rapid diagnosis. Many laboratories are able to provide diagnostic testing for influenza, parainfluenza (types 1–3), respiratory syncytial virus (RSV), and respiratory adenoviruses. A respiratory specimen containing cells is necessary for sensitive detection of viruses by immunofluorescence or other antigen-detection methods.

Good-quality diagnostic samples (often lavage or aspirate samples) can usually be obtained from young, immunocompetent, hospitalized individuals, but in other circumstances the ideal sample may not be available. Delays in transportation may reduce specimen quality and compromise assay results. The most-difficult (and least-efficient) specimens for diagnostic testing are swab samples containing minimal cellular material taken from largely asymptomatic individuals in the community. Smears from these samples can be difficult to interpret in a direct antigen test and thus culture of the sample is usually required for pathogen identification. Bacterial antigen detection by a direct fluorescent antibody (DFA) test is similarly compromised by poor sensitivity and has the added concern of artifacts leading to false-positive results, particularly for *B. pertussis* and *L. pneumophila*.

Serological assays of an antibody response to infection are available for some respiratory pathogens. Although useful for retrospective evidence of infection in a community, the results are not timely enough for patient management. For some cell-associated or intracellular bacteria and viruses, antibody responses develop slowly, if they develop at all, and convalescent sera taken many weeks after disease onset are required to make a definitive diagnosis (e.g., for *C. pneumoniae*, *Legionella* species, RSV). For other infections such as influenza, antibody responses are brisk, but frequent reinfection reduces the IgM response and convalescent sera are required to demonstrate changing titers.

Application of Molecular Assays to Respiratory Pathogen Diagnosis

The limitations of conventional testing are well recognized for viral^{11,12} and bacterial^{13,14} pathogens, and some of the

Table 41-3. Comparison of Nucleic Acid and Culture/Antigen Detection Methods for Respiratory Pathogen Diagnosis		
	Nucleic Acid Methods	Culture/Antigen Methods
Cost	Tests tend to be expensive (but getting cheaper)	Relatively inexpensive in laboratories already set up for these procedures, but “real” cost of maintaining cultures often underestimated
Speed	Rapid diagnostic methods	Speed very variable depending on the pathogen and method used
Infrastructure	Specialized laboratory set-up required	Specialized laboratory set-up required
Spectrum	Specific sequence information required for design; triage of testing can be difficult; generic primers may be used to identify novel pathogens	“Catch-all” approach, which may be advantageous when novel pathogens need to be identified
Sensitivity	Exquisite sensitivity but can be prone to cross-contamination problems	Generally less sensitive than nucleic acid detection methods
Specificity	Careful handling required to avoid contamination (common problem); primer/probe design crucial	Careful handling required to avoid contamination, but less-common problem than for molecular methods; DFA subject to over interpretation
Strain typing	Most definitive method	Limited serotyping (e.g., influenza, <i>Legionella</i>)
Automation	Automated extraction equipment becoming available; automated detection commonplace	Difficult to automate
Safety	Inactivated before analysis, but antimicrobial sensitivity information requires knowledge of genotypic mutation	Isolates useful for antimicrobial sensitivity testing and phenotyping, but specialized safety requirements needed for culture of category 3/4 pathogens
Quality assurance	Proficiency and validation of methods not well established	Culture depends critically on cell line or medium quality; maintaining quality can be difficult

key differences between nucleic acid and culture or direct antigen testing methods are summarized in Table 41-3. Utilization of rapid viral diagnostic procedures, such as may be provided by molecular amplification methods, could help to reduce the emergence of antibiotic-resistant bacteria. One study demonstrated that a 52% reduction in antibiotic use was possible using molecular methods for viral diagnosis.¹⁵

Despite the obvious economic burden of CAP, we do not have accurate data on how many of these infections are viral in origin or caused by the atypical bacteria for which routine diagnostic testing is not readily available. The identification of emerging human viral infections (such as H5N1 influenza and SARS-CoV) has heightened awareness of the gaps in respiratory pathogen diagnosis. Nucleic acid detection assays are likely to be utilized more widely to identify novel emerging pathogens that could result in worldwide outbreaks. Molecular amplification assays are being used successfully for the identification of organisms associated with pneumonia.¹⁶

The potential for nosocomial spread of respiratory pathogens is well recognized for patients admitted to the hospital with RSV, parainfluenza, or other infections, serving as a reservoir for transmission to vulnerable patients and leading to possible outbreaks.¹⁷ Previously, available diagnostic methods were not sensitive enough to identify sources of outbreaks, but the advent of molecular amplification methods has allowed even environmental sampling to be helpful in confirming outbreak sources and linking clustered cases of infection.¹⁸

Molecular Tests for Respiratory Pathogens

Samples and Nucleic Acid Extraction

Samples used for detection of respiratory pathogens include swabs (usually nasopharyngeal or throat), aspirates (nasopharyngeal or tracheal), sputum (usually from individuals presenting with pneumonia), or bronchoalveolar lavage specimens. For infections involving the entire respiratory tract, nasopharyngeal specimens are practical for diagnosis. For other infections, which are more focal,

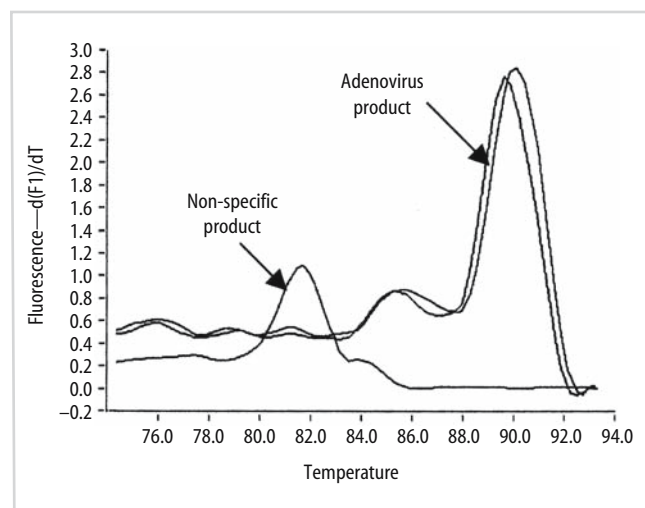


Figure 41-1. Melting-curve analysis of PCR products using the intercalating dye SYBR Green differentiates between specific and nonspecific products without further manipulation of the PCR products.

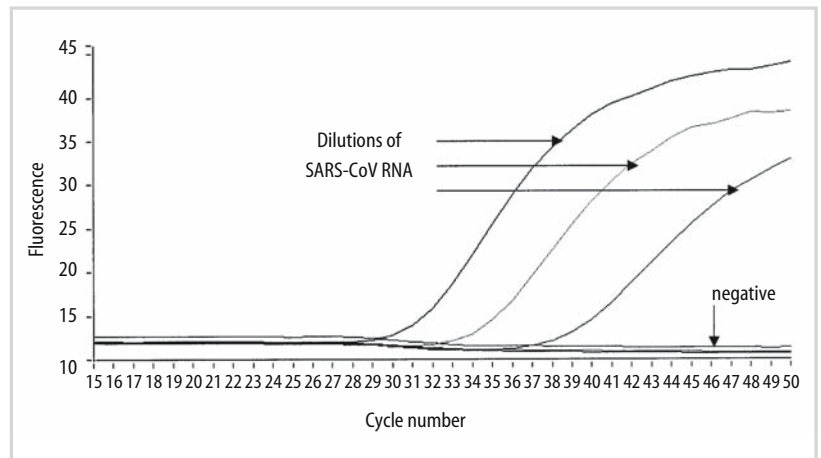


Figure 41-2. Detection of RT-PCR products for severe acute respiratory syndrome coronavirus (SARS-CoV) by a real-time PCR assay utilizing a hydrolysis (TaqMan) probe. The fluorescence signal is produced by measurement of the amount of fluorescent dye released each cycle.

lower respiratory tract specimens are required (e.g., for *Legionella*).

Sample preparation is a critical step for the detection and analysis of organisms. Numerous methods, from simple boiling to sophisticated automated protocols, are available for disruption of the organism and purification of the nucleic acids. Many studies have demonstrated inhibitors in respiratory specimens, making some form of extraction (with or without freezing) necessary to avoid frequent false negative results. Commercial kits for preparation of samples are available and should reduce interlaboratory variation in results. Simultaneous extraction of RNA and DNA facilitates assays for both viruses and bacteria.

Diagnostic Detection of Nucleic Acid

Molecular techniques for the detection and analysis of pathogens associated with respiratory infection provide specific diagnoses for individual cases and for outbreaks. Currently, FDA-cleared molecular tests are not available for

the detection of respiratory pathogens, with the exception of *Mycobacterium tuberculosis* (see chapter 43). Molecular tests are performed using either validated laboratory-developed procedures or commercial testing reagents. Published diagnostic methods for detection of respiratory pathogen DNA or RNA directly from clinical specimens utilize target amplification procedures such as polymerase chain reaction (PCR) or nucleic acid sequence-based amplification (NASBA). Although direct detection methods based on nucleic acid hybridization would be theoretically possible, the amount of target nucleic acid in specimens may be minimal and such methods would lack sensitivity compared to amplification methods, unless the organism was propagated before analysis. Thus, the molecular amplification procedures reported for direct detection of respiratory pathogens in clinical samples include PCR (e.g., Reference 19 and Figure 41-1), reverse transcription-polymerase chain reaction (RT-PCR) (e.g., Reference 20 and Figures 41-2 and 41-3), and NASBA (e.g., References 21–23 and Figures 41-4 and 41-5). Target nucleic acid for amplification assays usually is a pathogen-specific gene or genes from the pathogen genomic DNA or RNA, but some

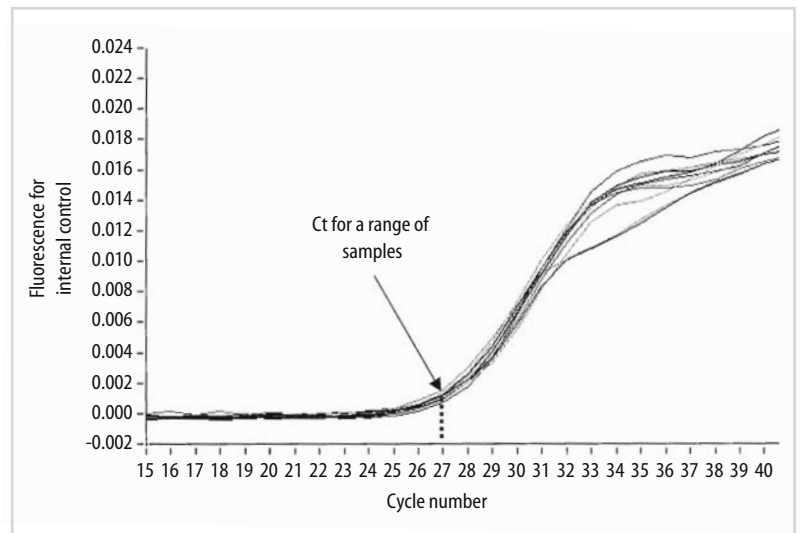


Figure 41-3. Analysis of internal control reactions for clinical samples in a real-time RT-PCR assay ensures that nucleic acid extraction of the sample was efficient and no residual inhibitors are present.

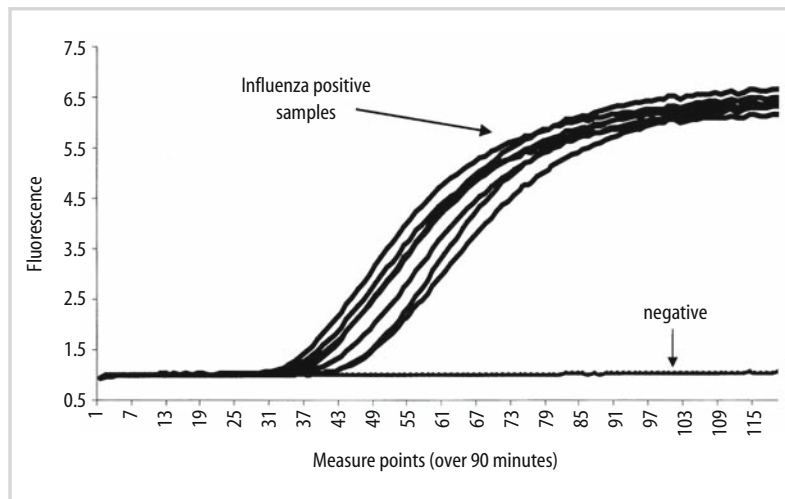


Figure 41-4. Real-time NASBA assay for influenza with detection of amplified products using a molecular beacon probe. Differentiation of positive from negative results is straightforward in this example.

assays have utilized bacterial ribosomal RNA (rRNA; e.g., Reference 22). For cellular samples tested for respiratory pathogens, targeting messenger RNA (mRNA) or genomic antisense RNA may enhance diagnostic sensitivity.

A variety of formats have been utilized for the detection of amplified products. Procedures that separate target amplification from the detection phase (agarose gel analysis or endpoint hybridization) are well established^{24,25} and may allow multiple targets to be analyzed in a single reaction, providing added typing information.

For ease of use and incorporation into diagnostic laboratories, most laboratory-developed assays for detection of respiratory pathogens utilize real-time amplification methods in which the amplification and detection steps are combined. Some methods use intercalating dyes with the analysis of PCR product melting temperatures (e.g., as described previously²⁶ and illustrated in Figure 41-1), whereas others use fluorogenic primers or probes (e.g., TaqMan, hybridization format, and molecular beacons^{19,21-23,27}) to ensure the specificity of the reaction. Figure 41-2 illustrates a real-time RT-PCR assay for SARS-CoV using probe-specific detection of amplified products (one fluorescence measurement per cycle). The range of real-time PCR or RT-PCR and NASBA methodologies used

for respiratory targets is diverse given the fact that assays are laboratory developed.^{28,29}

The difficulty with diagnosis of respiratory infections is the wide range of pathogens with similar presentations. The nucleic acid technologies utilized currently in the majority of diagnostic laboratories are real-time PCR single-target assays. In some cases, generic primers can be designed to pick up several related pathogens. Such generic primers may be based on conserved protein coding sequences (such as those essential for enzyme function) or noncoding regions for which variation is limited because of the need for maintenance of secondary structure. The use of primer sets to pick up genera or even families of organisms has shown promise in limited studies, including analysis of *Legionella* and *Mycobacteria*. Generic assays have the ability to detect many related organisms and may be used to characterize previously undescribed species in respiratory infection.

Limited multiplex procedures have been reported for detection of related organisms using real-time PCR or NASBA procedures, but such assays are difficult to set up and control. For many respiratory pathogens, there is sufficient variation that multiplex approaches have been developed to detect, for example, all possible respiratory

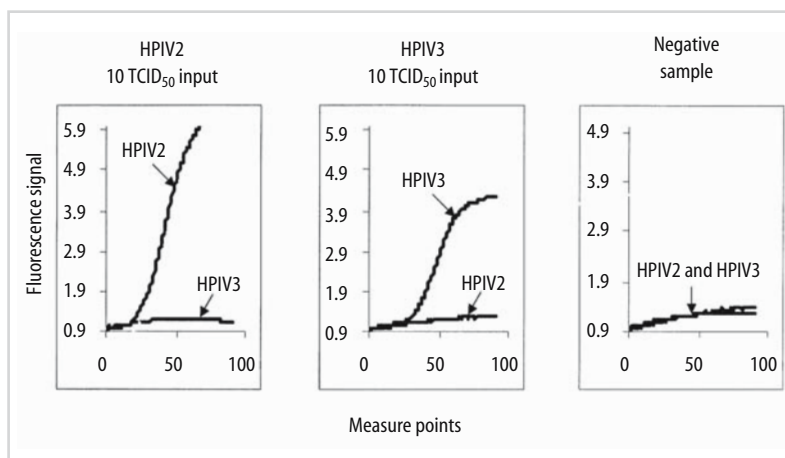


Figure 41-5. Multiplex real-time NASBA assay for human parainfluenza virus (HPIV) with simultaneous detection and differentiation of two target types using two sets of primers and specific molecular beacons labeled with FAM and ROX, respectively. TCID₅₀, 50% Tissue Culture Infecting Dose.

adenovirus,¹⁹ influenza,²⁰ or parainfluenza²¹ types. A simple, dual-labeled multiplex NASBA assay is shown in Figure 41-5 that uses separate primer sets and specific molecular beacon probes for parainfluenza types 2 and 3 (HPIV2 and HPIV3) in the same reaction mix. Each probe is labeled with a different fluorophore, allowing detection and differentiation of both viruses in a single reaction. An ambitious multiplex nested RT-PCR procedure with gel analysis of the amplicons detects influenza A, B, and C viruses, RSV (A and B subtypes), and adenoviruses in a single assay.³⁰ The procedure, while complex to set up and validate, was reported to have good specificity and better sensitivity than antigen/culture procedures.

Interpretation and validation of a negative result are important parts of diagnostic tests based on nucleic acid amplification. Some assays incorporate an internal control system to distinguish true-negative from false-negative results. The internal control may amplify with the pathogen-specific primers but result in an amplicon with a different size or internal sequence from the pathogen amplicon. Alternatively, the internal control may be an external sequence spiked into the reaction (heterologous control) and amplified with a primer set different from the pathogen primers. In the example shown in Figure 41-3, amplification of the RNA heterologous control is consistent across many clinical samples and ensures that there are no gross inhibitors present in the reactions. For cellular samples obtained for detection of respiratory pathogens, the internal control reaction can utilize human DNA, rRNA, or mRNA detection. Such approaches have the added value of assessing for sample collection and integrity, as well as amplification inhibitors.

The relative merits of commercial versus laboratory-developed tests depend on the laboratory facilities, the technical expertise available, and the clinical need for expanded diagnosis. Commercial testing reagents provide quality controls and procedure standardization that facilitate clinical studies.^{24,32} Many companies are focusing on providing analyte-specific reagents (ASRs) for respiratory pathogen assays. ASRs will provide the laboratory with quality-controlled primers and probes, while allowing

them the flexibility to test for currently known circulating pathogens or according to a local testing algorithm. Microarray-based detection of multiplexed PCR products also has been reported.³¹

Microbial Typing and Respiratory Outbreak Investigation

Classically, typing of bacteria or viruses has used serological techniques that rely on antibody-antigen interactions. One benefit of approaches based on DNA or RNA detection is the more-detailed, quantitative assessment of the relationship between organisms, providing valuable data relevant to outbreak investigations and community health. Variation at the nucleic acid sequence level is not necessarily reflected in altered protein sequence or function; thus, additional sequence variation information may not correlate with conventional typing methods.

Restriction fragment length polymorphism (RFLP) analysis by pulsed field gel electrophoresis (PFGE), either with or without blot hybridization, has been utilized for analysis of complex DNA genomes from a variety of respiratory pathogens. RFLP analysis also has been applied to PCR or RT-PCR products from respiratory bacteria and viruses. In general, such methods can provide resolution down to the subtype level and have proven useful in outbreak investigation, as illustrated in Figure 41-6 for *B. pertussis* isolates. The difficulty with gel-based typing assays, such as PFGE, is standardizing results and sharing data between laboratories. Amplified fragment length polymorphism (AFLP) analysis represents an alternative method with better discriminatory power and portability, but this approach has not been used extensively for respiratory isolates to date.

For respiratory viruses, other methods have been used for typing, including heteroduplex mobility assay (HMA), single-strand conformation polymorphism (SSCP), and RFLP analysis of amplified PCR products. In general, HMA is considered technically complex but has the capacity to distinguish viral quasispecies with >3% nucleotide

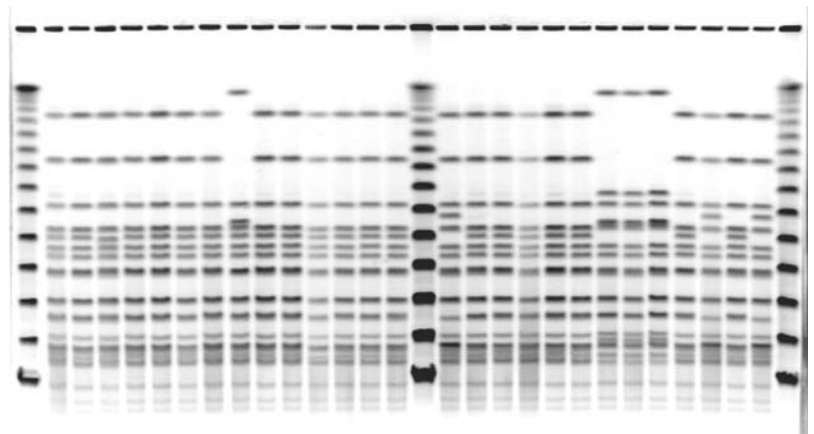


Figure 41-6. Pulsed field gel electrophoresis for analysis of *Bor-detella pertussis* isolates. (Figure kindly provided by Dr. M. Peppler, University of Alberta.)

differences. SSCP and RFLP, while technically easier, generally can resolve viruses only to the subtype level, and RFLP has the added constraint of assessing only sequence differences in restriction sites.

The use of sequencing to assess the relationship among viruses is well established, and molecular phylogenetic knowledge is expanding, allowing modeling of viral populations and prediction of new outbreaks.³³ The level of resolution using primary sequence is at one genome, and point mutations can be identified. Sometimes this provides more information than originally sought and creates problems in interpretation; while in other circumstances even small sequence variations can confer important changes in viral transmissibility and disease outcome. Identification of emerging viruses, which may have been recently introduced into the human population (e.g., SARS-CoV and influenza A H5N1 types), is critical to public health. Analysis of such novel viruses has relied heavily on sequencing of isolates or amplicons.^{9,10,34}

Human influenza A viruses are associated with enhanced morbidity and mortality compared with influenza B or influenza C viruses. Differences in pathogenicity for subtypes of influenza A also have been reported; for example, H3N2 is associated with more severe infection than H1N1. Such types and subtypes of influenza

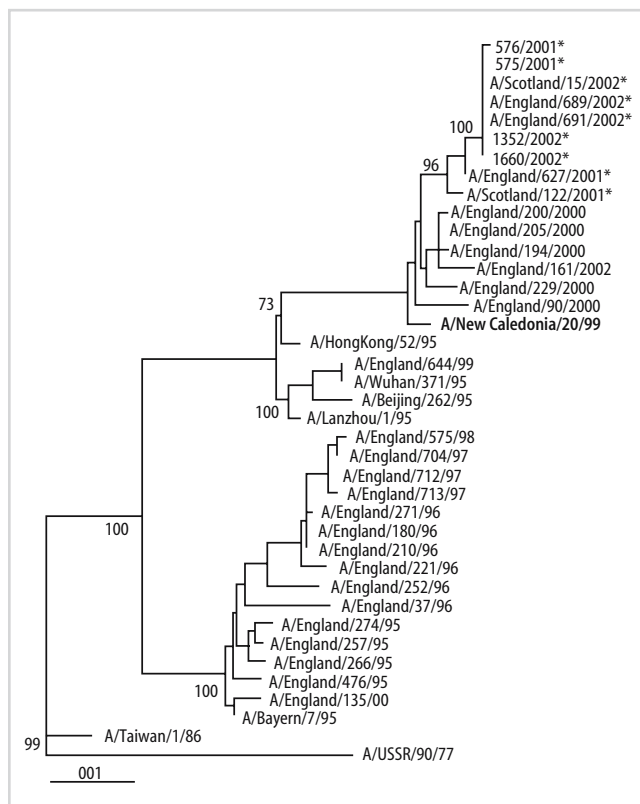


Figure 41-7. Sequence comparison of hemagglutinin (HA) for H1N1 and H1N2 viruses circulating in the United Kingdom in 2001 to 2002. The H1N1 vaccine strain is in bold typeface. H1N2 strains are indicated with asterisks. (Reproduced from Ellis JS, Alvarez-Aguero A, Gregory V, et al. Influenza AH1N2 viruses, United Kingdom, 2001–02 influenza season. *Emerg Infect Dis.* 2003;9:304–310.)

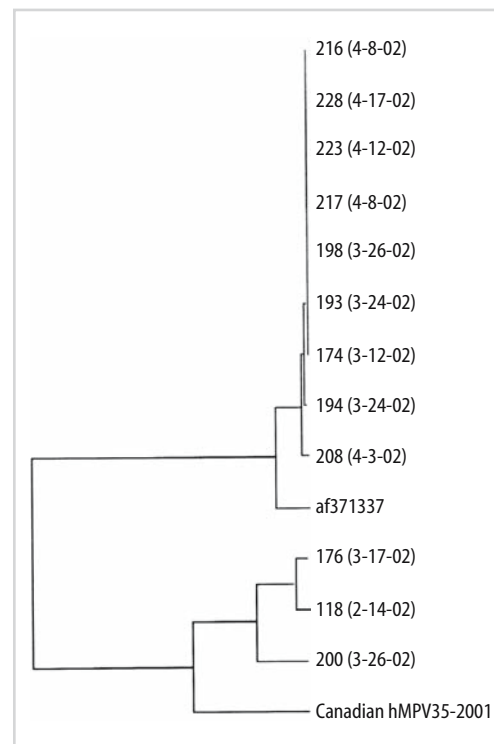


Figure 41-8. Phylogenetic tree illustrating the relationships among fusion gene sequences for 12 human metapneumovirus strains (from 2002). af371337 is the GenBank accession number for the prototype human metapneumovirus strain, and HMPV 35 is a Canadian strain isolated in 2001. (Reprinted from Boivin G, De Serres G, Cote S, et al. Human metapneumovirus infections in hospitalized children. *Emerg Infect Dis.* 2003;9:634–640.)

A can circulate independently, and their identification is important for assessment of current vaccine efficacy. Reassortment of the two predominant influenza subtypes infecting humans in recent times has been reported, and analysis of main hemagglutinin (HA) types has been undertaken by a range of molecular and nonmolecular methods.^{35,36} Detailed sequence comparison for HA of H1N1 and H1N2 viruses circulating in the United Kingdom in 2001–2002 (Figure 41-7 and Reference 37) illustrates the utility of sequence analysis in understanding viral divergence and relationship to current vaccine use. Reports of avian H5N1 viruses infecting humans (1997–1998 and 2003–2004^{34,38}) emphasize the need for detailed surveillance of influenza viruses and vigilance in identification of emerging viruses of importance to public health. Detailed analysis of avian H5N1 viruses that have infected humans to date have confirmed that all genes are of avian origin and are associated with minimal or very inefficient human-to-human spread. The potential for reassorted viruses that could more easily spread among humans is clear, and molecular methods are now an important part of influenza surveillance.

Recent studies of human metapneumovirus have identified two main lineages, with sequence diversity within each group (Figure 41-8⁴⁰), thus displaying a similar pattern to RSV isolates that are classified into two major

groups, A and B.³⁹ Further studies will confirm whether this distinction is associated with differences in virulence.

Sequence analysis for typing of bacteria has been slower to develop than that for viruses but has been utilized for investigation of some atypical bacteria associated with outbreaks of respiratory infection (e.g., *Legionella*⁴¹). Due to the problem of recombination, characterization of a single bacterial gene often does not reflect the organism as a whole. Multilocus sequence typing is a strategy that addresses this and appears useful for analysis of *B. pertussis*.⁴²

Microarray hybridization methods have been used to identify and differentiate related pathogens.³¹ Such an approach was useful in first identifying the agent of SARS as a coronavirus.⁴³ Molecular methods provide additional information about the virulence and type of infectious organism, as illustrated by recent experience with SARS-CoV and influenza types.

Interpretation of Test Results

Molecular tests have advantages over conventional procedures, but the sensitivity of molecular amplification methods can lead to problems with interpretation of results. For many organisms, a gold standard method is not available that accurately reflects the enhanced sensitivity of molecular methods, as has been seen with PCR testing for *B. pertussis* or *C. pneumoniae* in clinical samples. Studies confirm that PCR tests are very sensitive, and PCR-positive individuals may be culture negative or asymptomatic, so that results must always be interpreted in the clinical context.

Inhibitors of amplification are common in respiratory specimens, so a negative result must be interpreted in the context of the nucleic acid extraction method and the control results to monitor for nucleic acid degradation and amplification inhibition. When assays for the detection of respiratory pathogens are designed, primers and probes should not cross-react with normal respiratory flora or other respiratory pathogens.

Laboratory Issues

The triage of molecular testing for respiratory infection diagnosis is difficult. Currently, a single respiratory pathogen test detects only one or a few related pathogens. Also, bacterial testing and viral testing are not combined. Thus, many molecular tests must be used to screen for all appropriate pathogens, which increases testing costs. Thus, a laboratory that embarks on using molecular methods for the diagnosis of respiratory infections may require a complex testing algorithm. One approach is use a multiplex amplification procedure to identify multiple pathogens in a single assay, with certain assays now commercially available.^{24,32} Unfortunately, such tests tend to be expensive

and, if developed by the laboratory, are very difficult to control and ensure equal sensitivity and specificity for all pathogens. Thus, despite the potential for replacement of many culture and antigen procedures with nucleic acid amplification assays, such a molecular diagnostic revolution has not yet happened. The exception is for new pathogens when nucleic acid amplification and detection methods are clearly far superior to alternatives (e.g., metapneumovirus, SARS-CoV) or for testing of samples that are suboptimal for routine procedures (e.g., in surveillance situations).

Future Directions

Respiratory infections are currently underdiagnosed, despite the fact that accurate pathogen identification is important to ensure appropriate patient management and monitor infectious trends in the community. The major stumbling blocks in the diagnosis and investigation of respiratory infections are the complexity of testing algorithms and the number of potential targets that cause both upper and lower respiratory tract symptoms. Real-time PCR methods have vastly improved the sensitivity for detection and recognition of some difficult-to-culture organisms, and will likely become standard practice in the clinical laboratory in the next few years. There is, however, a limit to how many organisms can be “multiplexed” in a single test.

Microarray hybridization of randomly amplified PCR products from respiratory cultures and clinical samples has shown some success.³¹ If the promise of early experiments is maintained when applied to large-scale clinical studies, this could answer some of the technical problems surrounding the use of multiplex systems. Microarray hybridization, while not currently as convenient as real-time PCR detection methods, potentially has the benefit of being able to resolve complex product mixtures and provide clinically valuable information.

The use of molecular methods for typing and outbreak investigation of respiratory pathogens of public health importance is well established and is likely to expand. Future directions will incorporate the use of microarray systems for respiratory pathogen detection and analysis to allow crossing of the barriers between conventional virology and bacteriology (and mycology/parasitology). Once microarray systems have been developed and validated, the costs of this enhanced technology may be reduced and justifiable.

Identification of novel viruses, which have presumably only recently been introduced to humans, has reinforced the need for careful surveillance of emerging respiratory pathogens and institution of appropriate infection control measures. Lessons should be learned from the continuous sensitive surveillance and typing of organisms such as influenza and *B. pertussis* to direct the use and efficacy of available vaccines.

Molecular techniques developed for detection and analysis of the microbes responsible for respiratory

infections will be vital to our understanding of pathogenic mechanisms, appropriate management, and prevention of outbreaks in the future. Gel-based typing procedures (PFGE, HMA, SSCP) slowly will be replaced by sequence-based alternatives (e.g., multilocus sequence typing or MLST), which are more amenable to standardization and sharing of data among laboratories.

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