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Detection of Respiratory Viruses by Molecular Methods

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INTRODUCTION

Acute respiratory disease (ARD) accounts for an estimated 75% of all acute morbidities in developed countries, and most of these infections (approximately 80%) are viral. Upper respiratory tract infections (URTIs) such as rhinitis, pharyngitis, and laryngitis are among the most common infections in children, occurring three to eight times per year in infants and young children, with the incidence varying inversely with age, with young children having the higher frequency (23, 235). The Centers for Disease Control and Prevention's national vital statistics report indicates that there are between 12 and 32 million episodes of URTI each year in children aged 1 to 2 years (23). URTI can lead to acute asthma exacerbations, acute otitis media, and lower respiratory tract infection (LRTI) such as bronchitis, brochiolitis, and pneumonia. Acute viral respiratory tract infection is the leading cause of hospitalization for infants and young children in developed countries and is a major cause of death in developing countries (235, 293). In clinical practice, a specific virus is often not identified due to the lack of sensitive tests and/or the presence of as-yet-unknown pathogens (3, 87).

RESPIRATORY VIRUS PATHOGENS

The major causes of ARD in children and adults are influenza A and B viruses, parainfluenza virus (PIV) type 1 (PIV1), PIV2, PIV3, respiratory syncytial virus (RSV), adenovirus, and rhinovirus. Other viruses such as coronavirus (CoV), bocavirus, enterovirus, PIV4, the newly discovered parvovirus types 4 and 5, and mimivirus also infect the respiratory tract albeit at a much lower frequency, and the clinical importance of bocavirus, parvovirus types 4 and 5, and mimivirus is not known. Rhinoviruses and CoVs were identified as being human pathogens in the 1960s (269), but they have been largely ignored by the medical community because their clinical impact was considered to be minor. It is now clear that rhinoviruses and CoVs, once thought to cause only a common cold, can cause LRTI and ARD and can be fatal in some cases. Indeed, all of the viruses mentioned above have overlapping clinical presentations and cause both URTI and LRTI, and attending physicians usually cannot distinguish the causative agent without a laboratory diagnosis. Since 2000, newly discovered respiratory viruses including avian influenza viruses (H5N1, H7N7, and H7N3), human metapneumovirus (hMPV), severe acute respiratory syndrome (SARS) CoV, and human CoVs (HCoV) NL63 and HKU1 emerged. Throughout the 1990s, the approach to diagnosing respiratory virus infections continued to improve with the adoption of molecular testing. Nucleic acid amplification tests (NAATs) that first emerged in the 1980s for human immunodeficiency virus (HIV) and then for *Chlamydia trachomatis* were quickly applied to the diagnosis of respiratory viruses. It was the emergence of SARS-CoV in 2003 that showcased the importance of NAAT for diagnosing SARS infection. NAATs have now been developed for all respiratory viruses including both traditional viruses and emerging viruses. One final development, viz., multiplex amplification, would complete the transition from traditional diagnostic testing

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TABLE 1. Incubation period and mode of transmission for respiratory viruses

Virus	Incubation period (days)	Infectious period	Mode of transmission	Reference(s)
Influenza virus	1–4	7 days	Direct contact, aerosolized droplets	107
PIV	1–7	1–3 wk	Direct contact or aerosolized droplets	6, 110
RSV	2–8	3–8 days, up to 3–4 wk in infants	Direct contact or aerosolized droplets	7
Adenovirus	2–14	Days to mo	Direct contact or aerosolized droplets	5
Rhinovirus	2–3	7–10 days	Contact, droplet	61
hMPV	4–6	Mean of 5 days	Droplets	64
CoV (non-SARS)	2–4	Not known	Direct contact or aerosolized droplets	270
SARS CoV	2–10	Not known	Aerosolized droplets or fecal material	206
Bocavirus	Not known	Not known	Not known	
Parvovirus type 4/5	Not known	Not known	Not known	
Mimivirus	Not known	Not known	Not known, water droplet (?); *A. polyphagia*; may be vector	

methods to molecular testing methods. In this review, I will review the epidemiology of respiratory viruses and focus on the contribution that molecular diagnostics has made to our ability to detect these infections and to understand their epidemiology. A discussion of SARS-CoV will not be included in this review, as this virus has been reviewed recently by Cheng et al. (39).

In addition to their ability to cause a variety of ARD syndromes, respiratory viruses share a relatively short incubation period and mode of spread from person to person (Table 1). Transmission may be by direct contact with contaminated secretions inoculating the nasal and conjunctival epithelium or by aerosolized droplets. These modes of transmission are important parameters for the control of outbreaks in a variety of settings including day care for children, long-term care for the elderly, and patients in health care settings such as community or tertiary care hospitals. Our understanding of the epidemiology of respiratory virus infections is changing with the discovery of new viruses and the advent of newer, more sensitive molecular tests. Table 2 summarizes the prevalence of various respiratory viruses in studies conducted around the world. The wide range of prevalences for individual viruses likely reflects both the different seasons in which studies were conducted and the sensitivities of molecular tests used.

TRADITIONAL DIAGNOSTIC METHODS

Over the past two decades, virus isolation and serology have been the mainstay of the clinical laboratory for diagnosing respiratory virus infections. Virus isolation was performed using three or four cell lines and, together with embryonated hen eggs for influenza virus, provided the means for isolating respiratory viruses. A variety of serological tests including the hemagglutination inhibition (HAI) test, complement fixation, and enzyme immunoassay (EIA) were used for testing paired acute- and convalescent-phase sera for diagnosing infections, and in the case of influenza virus, HAI was able to subtype the virus as being H1 or H3 virus. Traditional tube cultures were routinely hemadsorbed with red blood cells on days 2 to 5 and day 10 to detect the presence of a "hemagglutinating virus" when the viral cytopathic effect (CPE) was minimal. In the early 1990s, tube cultures were replaced by shell vial culture (SVC) and, together with specific monoclonal antibodies, could detect specific viral antigens in 1 to 2 days instead of 8 to 10 days for tube culture. Direct fluorescent antibody (DFA) staining of cells derived from nasopharyngeal swabs or nasopharyngeal aspirates (NPA) became the mainstay for many laboratories and provided a rapid test result in about 3 h. EIAs were also introduced in the 1980s and 1990s, but these tests

TABLE 2. Prevalence of respiratory virus infections determined by molecular methods in several studies conducted in several countries

Agent	Prevalence (%)	No. of studies/ no. of countries	Reference(s)
Influenza virus	6–40	7/5	68, 87, 178, 269, 285, 286, 311
Avian influenza H5N1 virus	Sporadic outbreaks	1/1	306
PIV	15–30	4/4	6, 90, 101, 177
RSV	10–30	4/4	89, 90, 147, 177
Adenovirus	2–4	4/4	5, 88, 177, 288
HCoV-OC43	5–30	5/4	79, 90, 177, 207, 277
HCoV-229E	1–5	3/3	90, 177, 277
Rhinovirus/enterovirus	12–45	6/5	45, 62, 90, 177, 187, 284
hMPV	1.5–30	18/10	26, 28, 29, 52, 54, 59, 64, 65, 93, 99, 118, 147, 170, 174, 176, 224, 242, 280
HCoV-NL63	1.7–9.3	12/9	9, 16, 17, 41, 42, 64, 65, 128, 177, 190, 254
HCoV-HKU1	1–11.3	9/8	71, 73, 92, 96, 146, 155, 177, 242, 304
Bocavirus	2.1–11.3	14/12	3, 4, 10, 42, 129, 134, 167, 173, 177, 178, 195, 241, 244

lacked sensitivity and were usually relegated to point-of-care testing in defined settings. Nucleic acid amplification procedures including PCR, nucleic acid sequence-based amplification (NASBA), and loop-mediated isothermal amplification (LAMP) were developed for most respiratory viruses by the end of the decade, and today, these highly sensitive NAATs are starting to be used in the routine clinical laboratory for detecting respiratory viruses. Multiplex PCR coupled with fluidic microarrays using microbeads or DNA chips (oligonucleotides spotted onto a slide or chips) represents the latest diagnostic approach for the clinical laboratory and completes the evolution of diagnostics for respiratory viruses.

MOLECULAR DIAGNOSTIC METHODS: DETECTION OF SPECIFIC VIRUSES BY MOLECULAR METHODS

Influenza Virus

Influenza virus is a negative-sense single-stranded RNA virus belonging to the family *Orthomyxoviridae*. Influenza virus causes annual epidemics in temperate climates that are characterized by a sudden increase in febrile respiratory illness and absenteeism from school and the work place; the epidemic period is generally 3 to 8 weeks (12, 107). Pandemics are global epidemics that follow the emergence of a novel virus with a new combination of the hemagglutinin (HA) or neuraminidase (NA) gene, called genetic shift, that occurs when two different virus subtypes coinfect cells followed by genetic reassortment of HA and NA genes producing a new virus subtype (316). Influenza is a febrile illness characterized by fever; cough; upper respiratory symptoms including sore throat, rhinorrhea, and nasal congestion; and systemic symptoms including headache, myalgia, and malaise that results in a significant number of hospitalizations in all age groups (262). Since many respiratory viruses can present with similar signs and symptoms, it is impossible to differentiate one virus infection from another clinically. The clinician therefore relies on the laboratory to identify the virus. Many clinicians commonly diagnose patients syndromically with influenza or influenza-like illness without laboratory identification of a virus. The positive predictive value (PPV) of a clinical diagnosis of influenza virus infection in an adult case ranged from 18% to 87% compared with cases of laboratory-confirmed influenza virus infection (99). During periods of high influenza virus activity, a clinical diagnosis based on acute onset of high fever and cough can be highly predictive of influenza (PPV, 79% to 87%; negative predictive value [NPV], 39% to 75%). The consequences of not identifying influenza virus in a nursing home or on a hospital ward could be catastrophic. In the hospital setting, the identification of influenza virus is important, as appropriate infection control practices (droplet isolation precautions) are important in preventing outbreaks. Influenza virus outbreaks in a hospital can be devastating given the wide range of immunocompromised patients (cancer patients and transplant recipients) that are highly susceptible to life-threatening influenza virus infection. In either setting, specific antiviral agents such as M2 channel inhibitors (amantidine and rimantidine) or NA inhibitors (oseltamivir and zanamavir) can be prescribed; however, these drugs are effective only when given within the first 24 h following infection. For these reasons, the laboratory identification of influenza virus is of paramount importance.

Over the past two decades, several diagnostic approaches have been used for diagnosing influenza virus infection. Serological tests such as the HAI test have been used to detect seroconversion of influenza virus and influenza A virus infections as either H1 or H3; however, it is no longer used in the clinical laboratory to diagnose influenza virus infection. Nasopharyngeal swabs and NPA are the preferred specimens for influenza virus detection. Isolation of influenza virus was historically performed in embryonated hen eggs or tube cultures of primary monkey kidney, Madin-Darby canine kidney (MDCK), or A549 cells (48). CPE consistent with influenza virus can be visualized by light microscopy but is variable depending on cell types (12). The disadvantage of tube cultures is the time needed to obtain a positive result, usually 4 to 5 days but in some cases as long as 10 to 14 days (159). Neither tube culture nor SVC, which can provide a more rapid result in 18 to 24 h (described below), can detect inactivated virus. SVC employing centrifugation assisted in the inoculation of clinical specimens onto preformed monolayers of primary monkey kidney cells or MDCK cells, and immunofluorescence (IF) staining with monoclonal antibodies was introduced in the 1990s (182). Commercially available R-Mix cells (Diagnostic Hybrids Inc., Athens, OH), a mixture of A549 and Mink lung cells, have been used extensively by clinical laboratories for the detection of the eight viruses that are detected by DFA (84, 297). The advantage of SVC is that most viruses can be identified in 18 to 48 h, compared with 4 to 14 days for traditional tube culture (75). The disadvantage of SVC is that some viruses do not replicate robustly in R-Mix cells, and if viruses need to be recovered for further analysis, they need to be passaged in R-Mix Too cells or primary monkey kidney cells (228). The combination of DFA plus SVC yields approximately 5 to 15% more positive results than does DFA alone (159, 312). DFA is used to detect influenza A and B viruses; PIV types 1, 2, and 3; adenovirus; RSV; and, more recently, hMPV. The DFA test has been the work horse of the clinical laboratory for over two decades, and excellent monoclonal antibodies are available commercially. DFA assays take 2 to 3 h to perform, although some laboratories batch specimens and do not process them as they arrive, thus delaying the turnaround time for reporting results. DFA alone or in combination with SVC is not well suited for the detection of mixed viral infections, which are more effectively detected by NAAT (177). DFA for influenza virus has a sensitivity of 70 to 100%, a specificity 80 to 100%, a PPV of 85 to 94%, and an NPV of 96 to 100% compared to cell culture (305). A number of rapid EIAs for influenza A and B viruses have been introduced over the past 10 years (13, 36, 37, 94, 272, 305). These tests generally have sensitivities of 70 to 75%, significantly lower than those of tube culture, SVC, and DFA, but good specificities of 90 to 95%. Due to the low sensitivity of these tests, false-negative results are a major concern, and the PPV will decrease as the prevalence of infection decreases. These rapid EIAs should be evaluated in-house, and their performances should be monitored from season to season to ensure that they pick up circulating strains.

Molecular tests for influenza virus detection include reverse transcriptase PCR (RT-PCR), NASBA, and LAMP. In the case of RT-PCR, nucleic acid is reverse transcribed into cDNA

TABLE 3. Molecular tests for detection of influenza A and B viruses[a]

Assay format	Gene target(s)	Sensitivity (%)	Specificity (%)	Description	Reference(s)
RT-PCR	Influenza A virus M (401 bp), influenza virus B HA (767 bp)	ND	ND	First RT-PCR assay for detection of influenza virus types A, B, and C	313
Multiplex RT-PCR	M HA (944 bp), H3 (591 bp)	ND	ND	Detects influenza A and B viruses; sensitivity of 1–5 PFU; more sensitive than culture based on 619 specimens; performance not determined	66
Multiplex RT-PCR	M H1 (944 bp), H3 (591 bp)	92	84	PCR more sensitive than culture based on testing 1,033 specimens	311
Real-time RT-PCR	Influenza A virus M, influenza B virus HA	ND	ND	Detects both influenza A and B viruses; LLOD of 0.02 $TCID_{50}$; more sensitive than SVC based on testing 98 specimens	285
Real time NASBA	NP	ND	ND	NASBA assay using molecular beacons detecting wide range of influenza A virus subtypes; LLOD of 0.01 $TCID_{50}$; more sensitive than DFA and culture; tested 378 specimens; performance not determined	191
Real-time RT-PCR	A/NS-1 (192 bp), B/NS-1 (241 bp)	ND	ND	Detection by automated fluorescent capillary electrophoresis; tested 1,470 specimens; performance not determined; only 3 influenza virus-positive specimens	299
LAMP	M	ND	ND	LAMP correctly identified 22/22 selected positive specimens and 31/31 selected negative specimens; had lower analytical sensitivity than culture (LLOD of 10^{-3} PFU/reaction); did not test prospective specimens	213
LAMP	HA	91.0	ND	LAMP was less sensitive than culture; detected 71/78 culture-positive specimens out of 83 specimens evaluated	119
Multiplex real-time RT-PCR	NS (190 bp)	ND	ND	Detects influenza A and B viruses, RSV, PIV1 to PIV4 using two-tube amplification; LLOD of 0.1 $TCID_{50}$ for influenza A virus; tested 358 specimens	260
Real-time RT-PCR	Influenza A virus M, influenza B virus HA	ND	ND	RT-PCR was more sensitive than culture based on 72 specimens from stem cell transplant patients	286
Real-time RT-PCR	M	ND	ND	Tested 1,138 specimens; RT-PCR was13% more sensitive than DFA; performance was not determined	147
RT-PCR	NS (190 bp)	ND	ND	RT-PCR was more sensitive than culture and EIA for 150 specimens; performance was not determined	249
Multiplex RT-PCR	NS (190 bp)	ND	ND	Detects influenza A and B viruses; performance was not determined	211
RT-PCR	M H1, H3	98	98	MChip uses amplicon fragmentation, hybridization to a microarray containing 15 oligonucleotides; tested 102 samples by RT-PCR and culture	184
Multiplex RT-PCR	Proprietary	100 for Flu A, 100 for Flu B	99.4 for Flu A, 98.8 for Flu B	Compared ProFlu-1 assay to DFA plus culture for 353 pediatric specimens	157
RT-PCR	M HA, NA	ND	ND	Flu-Chip 55 low-density microarray detects H1, H3, and H5 subtypes in 11-h test with an overall accuracy of 72% based on testing 72 specimens	263
Multiplex RT-PCR	HA	96.4 for A, 91.5 for B	95.9 for A, 96.7 for B	Multiplex RT-PCR detected 19 respiratory viruses using microfluidic microarray and Luminex xMAP system; LLOD of 0.8×10^{-1} $TCID_{50}$/ml for influenza A virus and 6×10^{-2} $TCID_{50}$/ml for influenza B virus; evaluated by testing 544 specimens with discordant resolution by uniplex PCR	176, 177
Multiplex RT-PCR	Proprietary	ND	ND	ResPlex II detected 12 viruses compared with culture and TaqMan PCR using 360 frozen specimens; performance was not calculated	161
Multiplex RT-PCR	Proprietary	ND	ND	MultiCode-PLx detects 17 viruses using the Luminex xMAP system; 354 specimens were tested; more sensitive than DFA and culture for influenza A virus	200

[a] ND, not determined; M, matrix protein.

using virus-specific oligonucleotide primers or random hexamers. Random hexamers have the advantage of generating cDNA for multiple viruses present in a specimen and for use in virus discovery, as cDNA is more stable during storage than extracted RNA or intact clinical specimens. Several RT-PCR assays for influenza virus infection have been described since the first report by Zhang and Evans in 1991 (313) (Table 3). Several different gene targets have been used for amplification including the matrix, HA, and NS protein genes. All of these targets have both conserved and unique sequences, permitting their use in either consensus or subtype-specific (H1 or H3) virus detection assays. Different targets are required for the

detection of influenza B virus. Assays that detect all influenza A virus subtypes using the conserved matrix gene target have been reported, while others have been developed to distinguish between influenza A, B, and C viruses or between subtypes using HA gene targets (68). Nested PCR assays have been developed and in some cases provide an increased sensitivity over that of nonnested PCR (311); however, most clinical laboratories will not use nested PCR because the amplification work load is doubled and the risk for PCR contamination is dramatically increased. Real-time RT-PCR assays for influenza virus infection that offer results more quickly than endpoint assays have also been described and in many cases have sensitivities equal to or better than culture (285). Well-optimized RT-PCR assays are generally 5 to 10% more sensitive than culture for the detection of influenza virus and other respiratory viruses (147, 260) in hospitalized children <5 years of age (299), adults with community-acquired pneumonia (261), or stem cell transplant patients (276). The largest increase in sensitivity seen with PCR compared to DFA is for specimens that have a low viral load (147). Only a few reports have included the confirmation of positive results with a second test or the use a combined reference standard after resolving discordant results to calculate sensitivity and specificity. The true performances of many of these tests are therefore not known. NASBA and LAMP assays for the detection of influenza virus using the same targets for amplification have been developed. In one study, NASBA was more sensitive than DFA or culture, while LAMP appeared to have a sensitivity equivalent to that of culture (191).

Multiplex RT-PCR assays for the detection of influenza virus and a number of other respiratory viruses have recently been introduced, and these assays are also more sensitive than culture (176, 221, 260, 311). A number of primers and probes are commercially available as analyte-specific reagents (ASRs), for example, the GeneXpert influenza A and B virus test from Cepheid (Sunnyvale, CA). Recently, four commercial multiplex assays for the detection of influenza virus and other respiratory viruses have been introduced. These assays include the ResPlex II assay (Qiagen), the MultiCode-PLx RVP assay (EraGen Biosciences), the Seeplex RV assay (Seegene Inc., Seoul South Korea), the NGEN RVA ASR kit (Nanogen Inc., San Diego, CA), and the xTAG RVP assay (Luminex Molecular Diagnostics, Toronto, Ontario, Canada). Two multiplex assays have recently been approved by the FDA. The first is the ProFLu+ assay (Prodesse Inc.), which is a modification of the ProFlu-1 assay that detects influenza A virus, influenza B virus, and RSV (157). The second is the xTAG RVP assay, which is approved for the detection of 12 respiratory viruses and the first test to be approved for both the identification and subtyping of H1 and H3 influenza A virus. It has a sensitivity and a specificity of 96.4% and 95.9% for influenza A virus and 91.5% and 96% for influenza B virus, respectively, compared to DFA and culture (34). Compared with other diagnostic approaches, NAATs have the highest sensitivity, followed by SVC, DFA, tube culture, and EIA.

Avian Influenza Virus (H5N1)

In the last 25 years, over 15 new viruses that infect humans have been discovered, but none have the notoriety of avian influenza H5N1 virus, which was first detected in Hong Kong in 1997 (97). Following the emergence of SARS, global jurisdictions such as the World Health Organization (WHO) have mobilized and are on high alert for the next emerging pandemic strain of influenza virus. It has been estimated that an influenza virus pandemic could result in 200 million people infected, 90 million clinically ill people, and 2 million people dead in the United States alone (10a). The economic costs would total nearly $675 billion. This compares to an average epidemic or interpandemic influenza virus season, which results in >30,000 deaths and >100,000 hospitalizations (238).

The first introduction of avian influenza H5N1 virus into the human population occurred in Hong Kong in 1997 and resulted in death in 6 of 18 documented cases. Exposure to infected chickens proved to be an important risk factor in all cases (131). The culling of 1.5 million chickens in Hong Kong markets was successful in preventing further human cases. In late 2003, H5N1 returned, infecting and killing millions of wild and domesticated birds in Cambodia, China, Japan, Indonesia, Laos, Malaysia, Thailand, and Vietnam. Transmission to humans has continued to climb following these zoonoses and stood at 359 people infected with 229 deaths in 14 countries as of 5 February 2008 (306). Additional infections of domesticated fowl and, subsequently, humans have occurred with other avian influenza viruses, viz., H7N7 and H7N3 in The Netherlands and Canada.

The first cases of H5N1 avian influenza virus in Hong Kong were detected by virus isolation (253) and measurement of H5N1-specific antibody using HAI or HA neutralization. Katz et al. (131) demonstrated that the kinetics of the antibody response to H5N1 is similar to that of a primary response to seasonal influenza virus, with serum-neutralizing antibody appearing ≥14 days after the onset of symptoms. H5N1-specific antibody has also been measured by enzyme-linked immunosorbent assay (ELISA) and Western blotting using baculovirus-expressed antigens. ELISA was more sensitive than microneutralization for both immunoglobulin M (IgM) and IgG antibody (131), and microneutralization was more sensitive than HAI (227). The maximum sensitivity (80%) and maximum specificity (100%) were achieved using a combination of neutralization test and Western blotting for adults, but the maximum sensitivity (100%) was achieved for children by using a combination of ELISA and neutralization test (227). Rapid EIAs have been evaluated for their abilities to detect H5N1 (108). Reports indicated that the Directigen Flu A antigen ELISA (BD Diagnostic Systems, Sparks, MD) could detect H5N1, H7N2, and H7N3 (310). Fedorko et al. (82) evaluated the Directigen Flu A+B and Xpert Flu A&B assays (Cepheid, Sunnyvale, CA) for their abilities to detect H5N1 and H9N2 subtypes. Both assays had a lower limit of detection (LLOD) of 5×10^4 50% tissue culture infective doses ($TCID_{50}$) for both subtypes, but the Directigen Flu A+B assay failed to detect wild-type A/Hong Kong/491/1997 virus. Another commercial rapid EIA test, the Espline Influenza A&B Kit-N (Fujirebio Inc., Japan), detects nucleocapsid antigen for all 15 HA subtypes but has not been validated using clinical specimens (13). Chan et al. (36) compared the performances of six rapid influenza A virus tests for their abilities to detect H1N1, H3N2, and H5N1 viruses. The tests included QuickVue Influenza A+B (Quidel Corporation, San Diego, CA), Binax Now Influenza

A&B (Binax Inc., Scarborough, ME), Directogen Flu A+B, Directogen EZ Flu A+B, and Rapid Tests Flu II assays. All six kits were able to detect H5N1, but the LLOD for all of the rapid tests was 1,000-fold higher than that of culture (36). Those authors conclude that the low sensitivity of the rapid tests for detecting H5N1 is a limitation of the technology and not due to a difference in the H5N1 virus compared with H1N1 or H3N2. These rapid assays may miss a number of infections and therefore should be used with caution.

Several different molecular tests for the detection of influenza H5N1 virus including RT-PCR, NASBA, and RT-LAMP have been described (Table 4). Poddar described one-step RT-PCR assays that amplify the matrix, NP, and HA genes of influenza A and B virus, giving different-sized amplicons that could be separated by gel electrophoresis (212). Specific primers for different HA types have been multiplexed with matrix and NP primers for the detection and identification of H1, H3, or H5 virus subtypes. The assay had an LLOD of 0.01 $TCID_{50}$ for both influenza A and B viruses and showed good agreement with subtyping using monoclonal antibodies. Spackman et al. (245) developed a real-time RT-PCR for the detection of influenza virus subtypes H5 and H7 that had a sensitivity similar to that of culture. Valle et al. (276) developed a real-time assay for H5N1 that had a sensitivity of 1 PFU and was more sensitive than a nested PCR. Whiley and Sloots (300) described a real-time RT-PCR assay amplifying a conserved region of the matrix gene using an ABI 7500 instrument (Applied Biosciences Inc., San Diego, CA) that was able to detect a wide range of influenza A virus subtypes. This assay was 100% sensitive and 100% specific compared to an end-point assay. Ng et al. (196) described a real-time RT-PCR assay using the LightCycler apparatus and two primer pairs that were specific for the H5 HA gene. This assay was compared to a nested end-point PCR and the Binax Now rapid test using 28 known H5N1-positive specimens. RT-PCR was 3 logs more sensitive than the rapid test and detected all 28 positive specimens, compared to 25 detected by the nested assay. Ng et al. (197) developed a real-time RT-PCR assay that amplified a 456-bp fragment of the HA gene with an analytical sensitivity of 10^3 copies of H5N1. The assay reportedly had a 1- to 2-log-better sensitivity than the primers recommended by the WHO. They evaluated it using 145 specimens from chickens, ducks, and muscovies and demonstrated an overall sensitivity of 67% for cloacal and tracheal specimens compared to culture. Shan et al. (234) developed a NASBA procedure for H5N1 and showed that the sensitivity of NASBA was equivalent to that of egg culture for the detection of H5N1 in 94 avian blood samples and anal swabs. One of the NASBA primers targeted the polybasic amino acid sequence at the cleavage site of the HA0 hemagglutinin precursor, confirming the identification of a high-pathogenicity avian influenza (HPAI) virus. Jayawardena (121) described a LAMP assay for H5N1 that uses six oligonucleotide primers and had an LLOD of 2×10^{-2} PFU. This assay was not evaluated using human specimens and was not compared with any other amplification methods, so its performance characteristics are unknown. Wei et al. (296) described a multiplex RT-PCR amplifying the HA, N1, and NP genes for the detection and identification of H5N1 virus. This test was evaluated with only 10 isolates propagated in egg cultures. Steininger et al. (249) described an RT-PCR with a higher sensitivity (93%) for influenza A virus than for cell culture (80%) and ELISA. Xie et al. (309) developed a multiplex assay using four sets of primers to detect and identify three subtypes of influenza A virus, H5, H7, and H9. This end-point assay had a sensitivity of 100 pg for each influenza virus subtype. The assay was not evaluated using clinical specimens. Chen et al. (38) described a real-time assay to detect the HA gene of H5N1 that had an LLOD of 5×10^{-2} 50% egg infectious doses (EID_{50}) and 40 genomic copies, compared to 3 EID_{50} obtained using WHO primers and 10 EID_{50} obtained with an antigen capture ELISA. The assay detected 33 of 35 positive avian throat swabs and 60 of 60 positive human isolates. The RT-PCR was more sensitive than both culture and ELISA, with the latter detecting only 39% of the PCR-positive specimens. Ellis et al. (69) developed a real-time assay that was specific for A/H5 and could detect clades 1, 1′, 2, and 3 with an analytical sensitivity of <1 PFU for each clade. The 151-bp product amplified a fragment of the HA gene that contained an MseI restriction site, yielding 82- and 69-bp cleavage products that could be used for confirmation. Unfortunately, only 2 H5N1-positive and 15 negative specimens were tested by the assay, so performance is limiting. Hoffmann et al. (114) described a real-time assay that could detect the Qinghai-like HPAI virus lineage of H5N1 with one of the two primers homologous to the HA0 cleavage site. The assay was evaluated using 22 different H5 isolates, including H5N1, H5N2, H5N3, and H5N9, and only HPAI virus Qinghai-like isolates were positive. A recent real-time PCR assay for the detection of oseltamivir-resistant H5N1 was described by Chutinimitkul et al. (48). This assay detects the His274Tyr mutation in the NA gene that confers oseltamivir resistance, uses two TaqMan probes, has a sensitivity of 10 copies, and could detect mutant virus present in a mixture of wild-type and mutant virus cultures.

The only FDA-cleared test for the detection of H5N1 was approved in February 2006 and uses primers and probes developed by the CDC Network (35). This real-time RT-PCR test was developed for use by the 140 laboratories in 50 states making up the Laboratory Response Network as part of the pandemic preparedness plan. At the time of its approval, the test was shown to detect the Eurasian and North American lineages of H5N1, but performance data for real clinical specimens are limited. All assays for the detection of H5N1 are prone to missing clinical isolates due to sequence divergence within the amplified genes. For this reason, primers and probes need to be constantly evaluated against circulating strains.

Two new multiplex PCR assays that use microfluidic arrays and the Luminex xMAP system for the detection of H5N1 have been developed. The ResPlex III assay is a research-use-only (RUO) test developed by Genaco Biomedical Products Inc. (Huntsville, AL [now owned by Qiagen, The Netherlands]). The test is a nested RT-PCR assay that uses 10 primer pairs to detect the H1, H2, H3, H5, H7, H9, N1, and N2 genes (317). The gene-specific primers, which are chimeric and contain a universal sequence, are used to enrich the target copy number during the first few cycles. The inside gene-specific primers contain a tag sequence that is recognized by a universal set of SuperPrimers that then amplifies the product exponentially. Labeled PCR products are hybridized to a fluidic microarray of colored beads (Luminex, Austin, TX), each containing one oligonucleotide for one specific amplified product. The results

TABLE 4. Molecular tests for detection of avian influenza H5N1 virus[a]

Assay format	Gene target(s)	Sensitivity (%)	Specificity (%)	Description	Reference
Multiplex RT-PCR	H1, H3, H5 NP	ND	ND	Multiplex RT-PCR detection of H1, H3, and H5 using agarose gel detection; LLOD of 0.01 $TCID_{50}$	212
Real-time RT-PCR	H5, H7	ND	ND	Detects H5, H7; sensitivity similar to that of virus isolation; tested 1,550 avian tracheal and cloacal specimens	245
NASBA	HA0	ND	ND	Detects HPAI virus with a sensitivity equivalent to egg culture using 94 tracheal and cloacal specimens	234
Real-time RT-PCR	M	100	100	Sensitivity and specificity based on testing 126 retrospective specimens	300
Multiplex real-time RT-PCR	H5	100	ND	Uses 2 primer pairs; 10-fold more sensitive than nested assay; detected 18/18 positive specimens	196
LAMP	H5	ND	ND	Uses 6 primer pairs; detection limit of 2×10^{-3} PFU; sensitivity comparable to that of RT-PCR	121
Multiplex RT-PCR	NP (327 bp), N1 (405 bp), H5 (549 bp)	ND	ND	Detects H5 and N1 in single amplification; evaluated on only 10 egg isolates	296
Multiplex RT-PCR	Pan-HA (244 bp), H5 (860 bp), H7 (634 bp), H9 (488 bp)	ND	ND	Multiplex using 4 primer pairs to detect H5, H7, H9; evaluated using 37 avian specimens; not evaluated on human specimens	309
Real-time RT-PCR	H5 (130 bp)	ND	ND	More sensitive than virus isolation; LLOD of 40 GE; evaluated using 35 avian throat swabs and 60 human specimens	38
Real-time RT-PCR	H5 (151 bp)	ND	ND	Detects 4 H5N1 clades; LLOD of 1 PFU; uses RE cleavage site for confirmation; tested only 15 human specimens	69
RT-PCR	HA0 cleavage site	ND	ND	Sensitivity not determined; tested only 22 avian specimens	114
Real-time RT-PCR	HA	ND	ND	Detects His274Tyr oseltamivir-resistant mutants; LLOD of 10 GE	46
Multiplex RT-PCR	H1, H2, H3, H5, H7, H9, N1, and N2 NS genes for influenza A and B viruses	93	100	ResPlex III assay (Qiagen) uses Luminex xMAP system to detect H1, H2, H3, H5, H7, H9, N1, N2; LLOD of 1 $TCID_{50}$; 217 specimens evaluated	317
RT-PCR	HA	ND	ND	FluChip 55 low-density microarray detects H1, H3, H5 in 11-h assay with 72% accuracy determined by testing 72 specimens	264
Real-time RT-PCR	H5	ND	ND	Detects Asian lineage H5N1; approved by FDA for use by the Laboratory Response Network; performance not determined using clinical specimens	35
Real-time RT-PCR	M	ND	ND	Detection of influenza A, B, and H5N1 viruses; LLOD of 1 PFU; more sensitive than nested PCR; 250 clinical specimens evaluated	276
Real-time RT-PCR	M	97	100	MChip low-density microarray; fragmented amplicons are hybridized to immobilized oligonucleotides; less sensitive than RT-PCR; performance based on testing 43 isolates	56
MChip	H1, H3 M	98	98	MChip uses amplicon fragmentation and hybridization to a microarray containing 15 oligonucleotides; performance determined by testing 102 specimens	184
NASBA	HA0	ND	ND	Distinguishes between HPAI and LPAI H5N1 virus subtypes; detected 12/12 isolates; no data on clinical specimens; does not cross-react with H1, H3, H7, or H9	49
RT-PCR	H5 (456 bp)	ND	ND	Detects H5 but not H1, H3, H7, or H9; validated using 145 avian specimens; did not test human specimens	197

[a] ND, not determined; M, matrix; LPAI, low-pathogenicity avian influenza virus; RE, restriction endonuclease.

are analyzed using the Luminex (Austin, TX) X-Map system with the Luminex-100 instrument to detect specific viral genes. The assay, which detects both influenza A and B viruses, has a sensitivity of 1 $TCID_{50}$ for H5N1 and has been evaluated using 217 specimens. ResPlex III had a sensitivity of 93.3% and a specificity of 100% using a combined reference standard of RT-PCR, real-time PCR, and virus culture (317). The xTAG RVP test recently approved by the FDA for the detection of 12

respiratory viruses also detects influenza H5N1 virus with an LLOD of 0.4 fmol/reaction (143). Neither of these multiplex assays has been tested with sufficient clinical specimens to know their performance characteristics for H5N1.

Low-density DNA arrays and microchips have also been developed for influenza H5N1 virus. Townsend et al. (264) developed a low-density array for the detection and subtyping of H1N1, H3N2, and H5N1. In this 11-h assay, viral RNA is reverse transcribed and amplified by PCR, runoff transcripts are prepared, and the transcripts are fragmented for optimal hybridization to an array of immobilized oligonucleotides on glass slides (184). This FluChip-55 microarray was evaluated using 72 blind influenza virus specimens. The FluChip assay had a typing accuracy of 72% (264). Dawson et al. (56) described a single-gene microarray called the MChip for the detection and identification of H1N1, H3N2, and H5N1 subtypes. This assay is similar to the one described by Townsend but uses only 15 capture sequences targeting the conserved matrix gene and an automated image interpretation (artificial neural network) for pattern recognition. The MChip was evaluated blindly using 53 human specimens and correctly identified 92% of the specimens. In a separate report, this group evaluated the MChip assay by testing 102 respiratory specimens by MChip, culture, RT-PCR, and the QuickVue Influenza A&B rapid EIA (Quidel Corporation, San Diego, CA) (184). The MChip assays had the same sensitivity as SVC, with each assay detecting 57/102 specimens, but had a lower sensitivity than RT-PCR, which detected 61 positive cultures. In comparison, the QuickVue assay detected 53 positive specimens. The MChip assays therefore had sensitivities that were similar to those of other laboratory tests but took 7 h to complete.

PIV

PIV is a negative-sense single-stranded RNA virus belonging to the family *Paramyxoviridae*. There are four serotypes of PIV that infect humans (292). Their mode of spread and pathogenesis is similar to that of the influenza viruses; however, the genetic makeup is such that they do not undergo antigenic drift or shift. PIV1 is the major cause of acute croup in infants and young children but also causes mild URTI, pharyngitis, and tracheobronchitis in all age groups (158). Outbreaks in temperate climates tend to occur mostly in the autumn months. PIV2 is generally associated with lower infection rates than PIV1 or PIV3 and has been associated with mild URTI, croup in children, and, occasionally, LRTI. Infections occur predominantly in fall months. PIV3 is a major cause of severe LRTI in infants and young children, often causing croup, bronchitis, and pneumonia in children <1 year of age (307). In older children and adults, it can cause URTI or tracheobronchitis (180). Infections with PIV3 can occur in any season but are most prevalent in winter and spring months in temperate climates (6). PIV4 is the least common of this group and is generally associated with mild URTI. As a group, PIVs cause 15 to 30% of nonbacterial respiratory disease in children requiring hospitalization (101). The onset of illness can either be abrupt as an acute spasmodic cough or begin as a mild URTI evolving over 1 to 3 days to involve the lower tract. The duration of acute illness can vary from 1 to 3 weeks but generally lasts 7 to 10 days (6). PIV also causes LRTI in the elderly and immunocompromised patients including bone marrow recipients (171, 216).

PIV infections have historically been diagnosed by virus isolation, by detection of viral antigen or RNA by DFA and NAAT, respectively, or by serological tests such as HAI tests (159). Many commonly used cell lines support the growth of PIVs, including primary monkey kidney, LLC-MK2, Buffalo green monkey kidney (BGMK), A549, and MRC-5 cells (52). SVC using centrifugation-assisted inoculation of preformed monolayers of R-Mix cells and pre-CPE staining for viral antigens are widely used in clinical laboratories. Viral antigens are routinely detected in nasopharyngeal epithelial cells in many laboratories by DFA using a panel of monoclonal antibodies (151). The sensitivity of DFA compared to cell culture varies between laboratories depending on the reagents used and has ranged from 70% to 83% (143, 150). Dunn et al. (63) recently compared the sensitivity of DFA to that of SVC using R-Mix cells and showed that DFA had a sensitivity of 70.5%, while SVC had a sensitivity of 96.7% for seven respiratory viruses. SVC detected 33 (87%) of 38 PIV-positive specimens compared with traditional cell culture using four cell lines (BGMK, A549, MRC-5, and primary monkey kidney cells). Monoclonal antibody pools that use two fluorescent dyes can be used to detect PIV types 1, 2, and 3 (SimulFluor reagents; Chemicon International, Temecula, CA). These reagents are FDA approved for direct specimen testing and culture confirmation. SimulFluor reagents have excellent sensitivity and specificity compared with individual antibodies (151). PIV4 is not detected by most laboratories since specific antibodies have not yet been approved for antigen detection.

A variety of NAATs for detecting PIV have been described, and most have shown increased sensitivity compared to that of culture (Table 5). Fan and Hendrickson (80) described the first RT-PCR for PIV that had an analytical sensitivity of 600 copies/ml and was more sensitive than culture. The HA-NA gene has been the most common target for amplification and contains unique regions for the four serotypes (PIV1 to PIV4), allowing the development of serotype-specific assays (19). Nested and heminested RT-PCR assays have been developed, but these assays were evaluated with only a few positive specimens (62, 90). Multiplex RT-PCR assays for the detection and identification of PIV1 to PIV4 in a single assay have also been developed (90, 260). Templeton et al. (260) described a multiplex assay with an LLOD of 0.01 $TCID_{50}$ and showed that this assay was more sensitive than culture in an evaluation of 358 specimens. They unfortunately had only 11 positive samples and did not determine sensitivity and specificity. Bellau-Pujol et al. (19) also described a multiplex assay for the detection of PIV1 to PIV4 but detected only eight positive cultures in their study and did not determine performance characteristics. Kuypers et al. (147) developed a multiplex assay that targeted the polymerase and matrix genes. They evaluated this assay using 608 specimens and found the assay to be more sensitive than DFA. Other uniplex or multiplex PCR assays for the detection of PIV have been developed, and these assays consistently had equal or greater sensitivity than IFA or culture (19, 62, 90, 147). Fan et al. (81) evaluated a commercial multiplex assay that detects influenza A and B virus, RSV A and B, and PIV1 to PIV3. This commercial assay, the Hexaplex

TABLE 5. Molecular tests for detection of PIV[a]

Assay format	Gene target(s)	Sensitivity (%)	Specificity (%)	Description	Reference(s)
Real-time RT-PCR	HA-NA (180 bp)	100	95	PCR products detected by EIA; LLOD of 600 copies/ml; more sensitive than culture; performance based on testing 80 specimens	80
Multiple RT-PCR	Proprietary	ND	ND	Hexaplex (Prodesse) detects influenza A and B viruses, PIV1 to PIV4, RSV A and B; sensitivity similar to that of culture; tested 109 specimens; performance not determined	81
Multiplex RT-PCR	Proprietary	100	19	Hexaplex (Prodesse) detects influenza A and B viruses, PIV1 to PIV4, RSV A and B; performance determined by testing 363 specimens; only 4/23 PCR-positive specimens confirmed by culture	132
Multiplex RT-PCR	PIV1 to PIV3 HA-NA, PIV4 P	ND	ND	Detects PIV1 to PIV4; more sensitive than culture; LLOD of 0.01 $TCID_{50}$; tested 358 specimens, only 11 positive specimens	260
Multiplex RT-PCR	PIV1 (261 bp), PIV2 (340 bp), PIV3 (145 bp), PIV4 (380 bp) HA-NA	ND	ND	Detects PIV1 to PIV4; 203 specimens tested; only 14 positive results; performance not determined	21
Nested RT-PCR	L	100	ND	Sensitivity based on 4/4 positive specimens	62
Heminested multiplex RT-PCR	HA-NA	ND	ND	Detects PIV1 to PIV4; tested 263 specimens, only 8 positive results; performance not determined	90
RT-PCR	PIV1 *pol* (84 bp), PIV2 *pol* (78 bp), PIV3 M (66 bp)	ND	ND	More sensitive than DFA; tested 608 specimens by PCR; performance not determined	147
Multiplex RT-PCR	PIV1 to PIV3 HA, PIV4 P	100 (PIV1), 100 (PIV2), 84.2 (PIV3)	99.8 (PIV1), 99.8 (PIV2), 99.6 (PIV3)	Multiplex RT-PCR detects 19 respiratory viruses; uses fluidic microbead microarray and the Luminex xMAP system; LLOD of 1×10^{-3} $TCID_{50}$/ml to 10 $TCID_{50}$/ml; positive results confirmed by a second PCR and amplicon sequencing	176, 177
Multiplex RT-PCR	Proprietary	93.8 (PIV1), 72.6 (PIV2)	>99.1 (PIV1), >99.1 (PIV3)	ResPlex II detects 12 viruses compared to culture and TaqMan PCR using 360 frozen specimens	161
Multiplex RT-PCR	Proprietary	ND	ND	MultiCode-PLx detects 17 viruses using the Luminex xMAP system; 354 specimens were tested by DFA and culture; detected 2 out of 3 DFA- and culture-positive specimens	200

[a] *pol*, large polymerase gene; M, matrix gene; P, phosphoprotein gene; HA-NA, hemagglutinin-neuraminidase gene; ND, not determined.

assay (Prodesse, Milwaukee, WI), was compared to culture and evaluated using 363 specimens. Only 4 of 23 Hexaplex-positive specimens were confirmed by culture. In a second evaluation of the Hexaplex assay involving 109 specimens, Hexaplex had a sensitivity similar to that of culture (81). More evaluations of this assay will be required to know its true performance characteristics. Recently, four commercial multiplex assays for the detection of influenza virus and other respiratory viruses have been introduced; these include the ResPlex II assay (Qiagen), the MultiCode-PLx RVP assay (EraGen Biosciences), the Seeplex RV assay (Seegene Inc., Seoul South Korea), the NGEN RVA ASR kit (Nanogen Inc., San Diego, CA), and the xTAG RVP assay (Luminex Molecular Diagnostics, Toronto, Ontario, Canada). The xTAG RVP assay was 100% sensitive for PIV1 and PIV2, 84.2% sensitive for PIV3, and over 99% specific for all three types (34).

RSV

RSV is a single-stranded negative-sense RNA virus belonging to the genus *Pneumovirus* within the subfamily *Pneumovirinae* and the family *Paramyxoviridae*. RSV is the single most important etiological agent causing respiratory disease in infancy and is a major cause of bronchiolitis and pneumonia in infants under 2 years of age (7). There are two subtypes of RSV, RSV A and B, and infections with RSV A are thought to be more severe than those with RSV B. It has been estimated that over 100,000 hospitalizations and 4,500 deaths are related to RSV infection annually in the United States, with expenses in excess of 300 million dollars per year (103). Most children have been infected by the age of 3 years, and repeated infections are common (266). Disease often begins with rhinitis and progresses to bronchiolitis or pneumonia with cough, wheez-

ing, and respiratory distress, but as is the case with other respiratory viruses, it can present differently in children with previous exposure or who may be immunocompromised. The duration of illness is often 10 to 14 days, and the fatality rate for hospitalized infants is estimated to be 0.5 to 1% (7, 103). In some years, the number of RSV cases may approach the number of all other respiratory virus infections combined (82, 177). Most infections occur between late fall and early spring, with a peak prevalence in winter (103). The spread of RSV in the hospital setting can be a major problem compounded by a long period of virus shedding, usually 3 to 8 days, that may be as long as 3 to 4 weeks (7).

Historically, RSV has been more difficult to culture than other respiratory viruses because the virus is more labile, and specimens need to be kept cold. HEp-2, HeLa, and A549 cells are the most sensitive for primary isolation. The sensitivity of conventional tube culture compared with rapid antigen detection by IFA has varied from 57 to 90% (105, 133). The SVC method has shown increased sensitivity compared to that of tube culture and provided a shorter turnaround time for reporting results. The SVC method has been adopted by the majority of clinical laboratories in North America, and the sensitivity compared with those of DFA, EIA, or conventional culture has ranged from 67% to 92% (122, 214, 243). Commercially available R-Mix cells (Diagnostic Hybrids Inc., Athens, OH) have shortened the resulting turnaround time and increased the sensitivity over conventional culture (15, 63, 84, 250, 299). Many laboratories use a combination of direct antigen detection by DFA and SVC. The availability of excellent monoclonal antibodies (257) has increased the sensitivity of DFA to between 80 and 97% (133, 151, 214). Rapid EIAs have been used for many years for RSV detection. They offer the advantage of providing results in 15 to 30 min, but the downside of these tests is their poor sensitivity. Approximately a dozen rapid tests have been approved for RSV, and for the most part, their performances are similar; their sensitivities, however, are only 80 to 85% compared to those of DFA and or RT-PCR (27, 145, 219, 240, 314). The sensitivity of the second version of the Directigen RSV EIA has ranged from 61 to 86% (33, 201, 218). It should be pointed out that the performances of these rapid tests is only as good as the reference standard to which they are compared, and their performances will likely be even lower when they are compared to the most sensitive NAATs. In addition, the specificity of these tests will be much lower when they are used in summer months, when the prevalence is lower. Another cautionary note is that their sensitivities will be lower in adults than children since adults generally shed lower titers of virus than children (33, 78, 201, 298).

Sensitive and specific NAAT assays have been developed to address the poor sensitivity of conventional tests (Table 6). A variety of RT-PCR assays that use different formats for amplicon analysis including gel electrophoresis, restriction fragment length polymorphism, hybridization, sequencing, and EIA have been described. Targets for RT-PCR have included the fusion, nucleocapsid, and large polymerase subunit genes (Table 6). Many assays have been developed, and most are more sensitive than culture (113, 132, 162, 186, 256, 299). One assay targeting the fusion gene detected 122 positive specimens out of 668 specimens tested, compared to only 47 positive specimens detected by culture (299). Freymuth et al. (89) described an assay that detected both RSV A and B and used discordant testing to show that RT-PCR was 97.5% sensitive and 63.9% specific (89). A commercially available NASBA assay targeting the F gene (NucliSens Easy Q RSV A and B; bioMerieux) was recently evaluated using 508 specimens that were also tested by DFA and culture. NASBA was more sensitive than culture and DFA and had a sensitivity of 99% and a specificity of 87% (192). A number of multiplex assays that detect RSV plus a number of other respiratory viruses have been described. The first multiplex assays for RSV were less sensitive than IFA and culture (76). The Hexaplex assay (Prodesse Inc.) has been evaluated for RSV and had a sensitivity of 91% and a specificity of 98.6% in one study involving 254 specimens (113). In a second study involving 363 specimens, the Hexaplex assay had a sensitivity of 98.6% and a specificity of 97.9% (132). Syrmis et al. (256) developed a multiplex assay for the detection of seven respiratory viruses including influenza A and B virus, RSV A and B, PIV1 to PIV3, and adenovirus. This assay was more sensitive than culture and IFA for the detection of RSV when it was evaluated using 598 specimens and 123 RSV-positive specimens (256). The ProFlu+ assay (Prodesse Inc.) is a real-time multiplex assay for the detection of influenza A virus, influenza B virus, RSV A, and RSV B that has recently been FDA cleared. As mentioned above, four commercial assays detecting multiple viruses have been introduced. These include the ResPlex II assay (Qiagen), the MultiCode-PLx RVP assay (EraGen Biosciences), the Seeplex RV assay (Seegene Inc., Seoul, South Korea), the NGEN RVA ASR kit (Nanogen Inc., San Diego, CA), and the xTAG RVP assay (Luminex Molecular Diagnostics, Toronto, Ontario, Canada). The xTAG RVP assay has a sensitivity of 100% for RSV A and B and specificities of 98.4% and 97.4% for RSV A and RSV B, respectively (34, 176). The sensitivity and specificity of the MultiCode-Plx RVP assay relative to those of DFA and R-Mix culture for the detection of RSV were 91.7% and 99.4%, respectively (200). Several companies (Roche Diagnostics, Basel, Switzerland; Abbott Molecular Diagnostics, North Chicago, IL; Cepheid, Sunnyvale, CA; and Prodesse Inc., Waukesha, WI) have ASRs (primers and probes) for detecting RSV, but these tests have not been extensively evaluated, and their performance characteristics are largely unknown (81, 113, 132, 162). In summary, NAATs are the most sensitive method for detecting RSV, followed by DFA, SVC, tube culture, and EIA, which is the least sensitive.

Adenovirus

Adenoviruses are double-stranded DNA viruses belonging to the family *Adenoviridae*. There are at least 51 known serotypes of adenovirus, which are categorized into six subgenera (subgenera A to F). These viruses are ubiquitous, and infections are common. In addition to acute respiratory syndrome, adenovirus causes conjunctivitis, keratoconjunctivitis, and acute gastroenteritis. Serotypes 1 to 5, 7, 14, 19, and 37 infect the respiratory tract, causing a variety of mild symptoms including fever, rhinitis, pharyngitis, cough, and conjunctivitis and more severe disease including laryngitis, croup, bronchiolitis, or pneumonia, on occasion (5). Most adenovirus infections occur early in life, and by age of 10 years, most children have been infected with at least one serotype (88). The route of

TABLE 6. Molecular tests for detection of RSV[a]

Assay format	Gene target	Sensitivity (%)	Specificity (%)	Description	Reference(s)
RT-PCR	N (219–474 bp)	94.4	100	RT-PCR performance compared to culture using a total of 47 specimens and 18 positive specimens	258
RT-PCR	N (278 bp)	97.5	63.9	PCR detects both RSV A and B; more sensitive than IFA and culture; tested 238 specimens	89
Heminested RT-PCR	F (243 bp)	ND	ND	PCR more sensitive than culture and antigen detection based on testing 132 specimens	111
Multiplex RT-PCR		ND	ND	Hexaplex (Prodesse) detects influenza A and B virus, PIV1 to PIV3, RSV A and B	162
Real-time RT-PCR	F (242 bp), F (89 bp)	ND	ND	Real-time PCR using FRET probes was more sensitive than nested PCR based on testing of 71 specimens; LLOD of 145 pg	186
Multiplex RT-PCR	RSV A N (334 bp), RSV B N (183 bp)	ND	ND	Multiplex PCR uses 5 primer pairs; detects and subtypes influenza virus and RSV; LLOD of <1 PFU; tested only 65 RSV-positive specimens	251
Multiplex RT-PCR	L	100	100	Multiplex PCR detects seven viruses including influenza A and B viruses, PIV1 to PIV3, RSV, and adenovirus; more sensitive than culture and IFA based on testing 598 specimens including 123 RSV-positive samples	256
Multiplex RT-PCR	L (355 bp)	ND	ND	Multiplex PCR detects RSV A and B and PIV3 using 3 primer pairs; less sensitive then IFA and culture; evaluated using 261 specimens	77
RT-PCR	F (380 bp)	ND	ND	RT-PCR was twice as sensitive as culture detection; 122 vs 47 positive specimens; evaluated using 668 specimens	299
NASBA	F	99	87	NucliSens Easy Q RSV A & B (bioMerieux) was more sensitive than DFA and culture (131 vs 108); performance compared to culture in a retrospective study of 508 specimens	192
Multiplex quantitative RT-PCR	*pol* 1b N	ND	ND	Hexaplex multiplex PCR detects influenza A and B viruses, RSV A and B, PIV1 to PIV3 with a sensitivity of 5 GE	81
Multiplex RT-PCR	*pol* 1b N	91	98.6	Hexaplex evaluated for RSV had a sensitivity of 4,200 copies of RSV A and 42 copies of RSV B; performance based on testing 254 specimens including 44 DFA-positive specimens	113
Multiplex RT-PCR	*pol* 1b N	98.6	97.9	Hexaplex performance based on testing 363 specimens including 82 confirmed positive specimens by culture or Directigen RSV EIA	132
Multiplex RT-PCR	Proprietary	97.8	97.0	Compared ProFlu-1 assay to DFA and culture using 353 pediatric specimens	157
Multiplex RT-PCR	L	100 (RSV A), 100 (RSV B)	98.4 (RSV A), 97.4 (RSV B)	Multiplex RT-PCR detected 19 respiratory viruses using fluidic microbead microarray and the Luminex xMAP system; LLOD of 6×10^{-2} $TCID_{50}$ for RSV A and 6 $TCID_{50}$ for RSV B; positives were confirmed by second PCR and amplicon sequencing; performance based on 554 specimens tested	34, 168
Multiplex RT-PCR	Proprietary	73.3	>99.1	ResPlex II detected 12 viruses compared to culture and TaqMan PCR using 360 frozen specimens	161
Multiplex RT-PCR	Proprietary	ND	ND	MultiCode-PLx detected 17 viruses using Luminex xMAP system; 354 specimens tested by DFA and culture; detected all 12 DFA- and culture-positive specimens	200

[a] ND, not determined; L, large polymerase subunit, N, nucleocapsid protein; F, fusion protein; FRET, fluorescence resonance energy transfer.

entry for adenovirus is usually by inhalation of droplet nuclei or by the oral route. Infection with serotypes 1, 2, and 5 are most frequent in the first years of life, and all serotypes can occur during any season, but infections are most frequent in late winter and early spring. Historically, 1 to 5% of all respiratory infections have been caused by adenovirus (5). The prevalence of adenovirus respiratory infection in children has ranged from 2 to 14% (88, 288). Adenovirus also causes 5 to 15% of acute diarrheal infections in children. Following infection, adenovirus can remain in the host for periods ranging from days to several years (291). Adenoviruses are increasingly important in both solid-organ and bone marrow transplant patients (255, 315), and infections with adenovirus in pediatric transplant patients can exceed 22% with mortality rates as high as 60% (112). Adults can shed adenovirus from the upper respiratory tract for up to a week and from the eye for several weeks (5). Children shed nonenteric adenovirus types 40 and 41 for 3 to 6 weeks in the throat or stool following respiratory tract infection (112). The persistence of adenovirus and prolonged shedding mean that diagnostic approaches must be capable of detecting low levels of virus in clinical material.

Adenovirus infections can be diagnosed by a variety of traditional and molecular methods. Serological assays have been used over the years to measure adenovirus-specific IgM or rising levels of IgG antibody; however, serology is no longer for diagnosis because it is slow and less specific than antigen or DNA detection. Adenovirus has been detected by electron microscopy (EM), but EM is insensitive since 10^6 virions/ml are required for visualization, and EM is therefore not used to detect adenovirus in clinical specimens, with the exception being stool specimens, where the amount of virus is high and readily detected by EM. Adenovirus antigen can be detected by DFA, but this method has poor sensitivity compared to that of other respiratory viruses, and DFA is often combined with SVC to improve sensitivity. Adenovirus grows well in a variety of heteroploid epithelial cell lines, and SVC using commercially available R-Mix cells can be stained for antigens at 18 to 48 h using monoclonal antibodies available from a number of suppliers (74, 220, 284). In one study, the detection of adenoviruses in SVC stained at 2 days was only 50 to 85% sensitive compared to that of tube culture at 14 days, suggesting that 5 days should be used for the optimal detection of adenovirus in shell vials (220).

Molecular methods provide the most sensitive method for detecting adenovirus in clinical material, although few direct comparisons of different methods have been performed. Several end-point and real-time PCR assays for adenovirus have been described (Table 7). Most of these assays amplify conserved regions of the hexon gene; however, assays that target the fiber or VA RNA gene for amplification have been developed for typing (47, 67, 109, 202, 205, 275). Echavarria et al. (67) described a PCR targeting the hexon gene that had an LLOD of 0.2 PFU or 10 genome equivalents (GE) that could detect 18 genotypes in urine specimens (67). Vabret et al. (275) described assays targeting the VA and hexon genes. The VA PCR assay was more sensitive than the hexon assay, and both assays were more sensitive than culture when they were evaluated using 362 nasal specimens. Real-time PCR assays that are more sensitive than end-point assays that detect all 51 genotypes have been described (109) and have an internal amplification control (47). Heim et al. (109) described a qualitative PCR that detected all 51 genotypes, had an LLOD of 15 GE, and had a sensitivity of 98.1%, which was higher than that of culture. A multiplex PCR assay that detects both adenovirus and PIV1 to PIV3 with a sensitivity of 0.2 $TCID_{50}$ and that was more sensitive than culture and IF staining was described (202). Adenovirus can be detected by three commercial multiplex PCR assays that detect up to 19 respiratory viruses. The MultiCode-Plx assay (EraGen), the ResPlex III assay (Millipore), and the xTAG RVP assay (Luminex Molecular Diagnostics) all detect adenovirus, but these assays have only recently been introduced, and their performance for detecting adenovirus is largely unknown. Although there are several commercially available probes and primers for adenovirus that are being sold as ASRs, the xTAG RVP assay is the only FDA-approved molecular test for adenovirus and has a sensitivity of 78.3% and a specificity of 100% (34). In summary, molecular tests provide the highest level of sensitivity for detecting adenovirus, followed by tube culture, SVC, and DFA.

Rhinovirus

Rhinoviruses are positive-sense single-stranded RNA viruses belonging to the family *Picornaviridae*. Rhinoviruses are perhaps the most interesting of the conventional viruses given recent reports of higher-than-expected infection rates in hospitalized children with acute LRTI (8, 45, 62, 90, 165, 187). Rhinoviruses were once thought to cause only "the common cold" and ergo were neglected by the medical community until recently, when their clinical spectrum of disease expanded. There are more than 200 serotypes of rhinovirus, and they differ from enteroviruses in their acid lability (enterovirus survives the pH of the stomach, while rhinoviruses do not) and their preference for growth in cell culture at 33°C and not 37°C. Rhinovirus infections occur year round, with peaks in late spring and early September in temperate climates (61). Rhinovirus is transmitted either by direct contact with contaminated fomites followed by self-inoculation of the eye or nose or by aerosolized droplets (194). Virus replicates primarily in nasal epithelial cells, and the shedding of virus coincides with acute rhinitis and may persist for 1 to 3 weeks. Rhinoviruses cause approximately two-thirds of cases of the common cold and are probably responsible for more human infections than any other agent (61). Rhinoviruses have been associated with asthma exacerbations and decompensation in chronic lung disease (95, 137), sinusitis, and otitis media (199, 204, 268) and, as mentioned above, cause serious LRTIs and wheezing in young children (95, 126, 140, 187, 203, 267), adults (166, 198, 265), and immunocompromised individuals (44, 144, 215, 286). Rhinovirus viremia has been detected by RT-PCR in 11.4% of young children and 25% of children with rhinovirus-associated asthma exacerbation (140, 187, 308). An outbreak of rhinovirus infection with an unusually high mortality rate in a long-term care facility in Santa Cruz was recently reported (135). Rhinoviruses are often the more prevalent virus detected in children with acute respiratory disease (45). In a prospective multicenter surveillance study of hospitalized children <5 years of age presenting with ARD, Miller et al. (187) recently reported that rhinovirus was the number one respiratory virus detected. In a longitudinal study of young children, Winther et al. (302)

TABLE 7. Molecular tests for detection of adenovirus[a]

Assay format	Gene target(s)	Sensitivity (%)	Specificity (%)	Description	Reference
PCR	H (300 bp), H (139 bp)	ND	ND	Conventional PCR detects at 18 adenovirus genotypes in urine specimens; LLOD of 0.2 PFU or 10 GE	67
Multiplex RT-PCR	H (215 bp)	0	94	Multiplex PCR uses 5 primer pairs to detect adenovirus types 1–7, PIV1 to PIV3; LLOD of 0.2 $TCID_{50}$ for adenovirus; more sensitive than culture and IF staining using 112 specimens stored for a year	202
Real-time Q-PCR	H	98.1	ND	Uses consensus primers and detects all 51 genotypes; LLOD of 15 GE; quantitative over 7 logs; more sensitive than conventional PCR assay determined by testing 234 specimens	109
Real-time Q-PCR	H (72 bp)	ND	ND	Tested only 50 BAL specimens; performance not determined	160
Multiplex PCR	Fiber	ND	ND	Multiplex PCR targeting fiber gene uses 6 primer pairs and detects 6 subgroups, subgroups A to F, using microtiter plate EIA detection; LLOD of 10^{-2} to 2 $TCID_{50}$; validated using spiked specimens	205
Q-PCR	H	ND	ND	Q-PCR was used to quantitate adenovirus in blood and throat swabs; PCR was more sensitive than culture; tested 38 specimens, with only 8 positive specimens	236
PCR	H (161 bp), VA (240–290 bp and 490–520 bp)	97.9	93.2	Used VA gene primers to detect 7 different genotypes; VA-targeted PCR was more sensitive than hexon PCR; PCR was more sensitive than culture and IF staining based on testing 362 nasal specimens	275
Real-time Q-PCR	H (137 bp)	ND	ND	Multiplex PCR coamplifies internal control; detects all 51 serotypes	47
Multiplex PCR	H	78.3	100	Multiplex RT-PCR detects 19 respiratory viruses using fluidic microbead microarray and the Luminex xMAP system; LLOD of 40 $TCID_{50}$/ml; positive results confirmed by a second PCR and amplicon sequencing; performance based on 554 specimens tested	176
Multiplex RT-PCR	Proprietary	ND	ND	MultiCode-PLx detects 17 viruses using the Luminex xMAP system; 354 specimens tested by DFA and culture; 1 positive specimen detected by DFA and culture	200

[a] ND, not determined; H, hexon protein; VA, VA RNA gene; BAL, bronchoalveolar lavage; Q-PCR, quantitative PCR.

recently reported that that the average number of rhinovirus infections per year was 6 and that 20% of infections were asymptomatic. Given the high number of rhinovirus infections per year, a large proportion of which are asymptomatic, and a prolonged period of shedding in children, caution must be exercised when interpreting a causative role in children presenting to the hospital with ARD.

Rhinoviruses have traditionally been isolated in cultures of human diploid fibroblast cell lines such as WI38 cells (11). Most clinical laboratories have historically not diagnosed rhinovirus infections for several reasons. First, rhinovirus infections were not considered to be clinically important by infectious disease clinicians. Second, the culture of rhinovirus requires a second incubator for virus isolation at 33°C. The range of susceptibility of different cell lines and even different lots of the same cells made rhinovirus isolation even more difficult, as more than one cell line was often required for optimal sensitivity (11, 150). Third, since rhinoviruses were essentially neglected by the medical community, neither virologists nor the industry saw the need to develop monoclonal antibodies to assist with diagnosis. Fourth, rhinovirus serotypes lack a common group antigen, making the possibility of broadly reacting antibodies unlikely.

The detection of picornaviruses including rhinoviruses and enteroviruses is well suited to nucleic acid amplification methods since both contain a highly conserved 5′-noncoding region (NCR), providing an excellent target for amplification. RT-PCR and NASBA assays for rhinovirus detection have been described (Table 8), and these NAATs have been more sensitive than culture (22, 104, 117, 165, 248, 290). NAATs targeting the 5′-NCR are the most sensitive for the detection of rhinoviruses, but these assays also detect enteroviruses and may not be able to distinguish between the two groups of viruses. This can be achieved by use of a second enterovirus-specific PCR, design of primers with a unique restriction enzyme site, hybridization with unique probes, or use of nested PCR. PCR assays targeting the VP1 or VP4 genes have been used for distinguishing rhinovirus from enterovirus (135). In one study comparing RT-PCR, NASBA, and culture using 517 consecutive specimens from hospitalized children in Belgium, NASBA and RT-PCR produced comparable results, and both were more sensitive than culture, picking up almost twice as many

TABLE 8. Molecular tests for detection of rhinovirus[a]

Assay format	Gene target(s)	Sensitivity (%)	Specificity (%)	Comments	Reference(s)
RT-PCR	5′-NCR (120 bp)	85	64	RT-PCR detected 111 positive specimens out of 203 specimens tested; sensitivity was twice that of roller tube culture; performance determined using culture as reference test	22
RT-PCR	5′-NCR (120 bp)	97.2	80.8	RT-PCR used rhinovirus-specific primers and detected 42 positive specimens; sensitivity was 1–10 GE; performance determined using culture as reference standard	104
RT-PCR	5′-NCR (120 bp), VP2 (530 bp)	98	ND	RT-PCR was 98% sensitive compared to 66% for virus isolation; performance based on 71 positive specimens	117
Nested RT-PCR	5′-NCR (120 bp)	100	ND	RT-PCR detected 52/52 culture-positive specimens plus an additional 124 positive specimens that were not confirmed	248
RT-PCR	5′-NCR (120 bp)	84.4	83.5	RT-PCR performance determined using culture as reference standard; discordant analysis not performed	290
RT-PCR	5′-NCR	100	ND	RT-PCR used TaqMan probes specific for rhinovirus; RT-PCR detected all 7 culture-positive specimens plus 14 additional specimens; discordant analysis not performed	57
Real-time RT-PCR	5′-NCR (120 bp)	100	ND	Real-time RT-PCR was equally as sensitive as end-point assay and more sensitive than culture; LLOD of 0.09–0.9 fg RNA; all 38 culture-positive results were positive by RT-PCR	130
Nested multiplex RT-PCR	5′-NCR-VP1 (295 bp)	ND	ND	Multiplex RT-PCR had a sensitivity similar to that of uniplex PCR assays (<0.7 logs); evaluation performed with only 22 positive specimens	102
Real-time RT-PCR	5′-NCR-VP4 (380 bp)	72	ND	Real-time LightCycler assay using TaqMan probe was 10-fold-more sensitive than end-point assay and more sensitive than culture (72% vs 39%); performance based on only 18 positive specimens	53
Multiplex real-time RT-PCR	5′-NCR (142 bp)	ND	ND	Multiplex RT-PCR employed coamplification of internal control; LLOD of 0.01 $TCID_{50}$	230
Multiplex heminested RT-PCR	5′-NCR (450 bp)	86.7	ND	Three multiplex RT-PCR assays detected 12 respiratory viruses; RT-PCR detected 13/15 culture-positive rhinoviruses	18
NASBA RT-PCR	5′-NCR (380 bp)	85.1 (NASBA), 82.9 (RT-PCR)	ND	Both NASBA and RT-PCR were more sensitive than culture (85.1 and 82.9 vs 44.7); performance based on 93 positive specimens using an expanded reference standard	165
Multiplex MassTag RT-PCR	5′-NCR	ND	ND	Multiplex PCR uses 22 tagged primer pairs to identify 22 bacterial and viral pathogens; amplicons are photocleaved, and products were identified by mass spectrometry	149
Multiplex RT-PCR	5′-NCR	100	91.3	xTAG RVP detects 19 respiratory viruses using fluidic microbead microarray and the Luminex xMAP system; LLOD of 3×10^{-2} $TCID_{50}$/ml; positive specimens confirmed by a second PCR and amplicon sequencing; performance based on 554 specimens tested	34, 176
Multiplex RT-PCR	Proprietary	ND	ND	ResPlex II detects 12 viruses; detected 31 positive specimens (8.6%) out of 360 specimens	161
Multiplex RT-PCR	Proprietary	ND	ND	MultiCode-PLx detects 17 viruses using the Luminex xMAP system; 354 specimens tested by DFA and culture; detected 16 positive specimens out of 354, and 13 were confirmed by uniplex PCR	200

[a] ND, not determined; VP, viral capsid protein.

positive samples as culture (165). Blomqvist et al. (22) described an RT-PCR assay that detected twice as many rhinoviruses as tube culture. Another RT-PCR targeting the same 5′-NCR had a sensitivity of 98%, compared to only 66% for culture (117). Real-time assays with an LLOD of <1 fg RNA (130), including a LightCycler assay using TaqMan probes, have been developed, and these assays have been more sensitive than end-point assays (53). Multiplex assays, some employing internal amplification controls (230), that are equally as sensitive or more sensitive than uniplex assays have been developed (102). One NASBA assay that was more sensitive than RT-PCR and culture has been described (165). A MassTag PCR test capable of detecting 22 respiratory viruses including rhinoviruses and bacterial respiratory pathogens has been described (30). MassTag uses multiplex PCR in which the viral targets are encoded by a library of up to 64 distinct MassCode tags. The targets are amplified by primers labeled by a photocleavage link to molecular tags of different molecular weights. After the removal of unincorporated primers, the tags are released by UV irradiation and analyzed by mass spectroscopy. This test was recently used to identify the etiological agent during a respiratory outbreak of unknown origin in New York state in 2004 to 2005, identifying 26 of 79 unresolved infections as being caused by rhinovirus (149). Four recently introduced commercial multiplex assays can detect picornaviruses; these include the ResPlex II assay (Qiagen), the MultiCode-PLx RVP assay (EraGen Biosciences), the Seeplex RV assay (Seegene Inc., Seoul, South Korea), and the xTAG RVP assay (Luminex Molecular Diagnostics, Toronto, Ontario, Canada). The xTAG RVP assay is the only FDA-approved test for rhinovirus detection and was 100% sensitive and 91.3% specific in clinical evaluations (34). Some ASR tests are commercially available, but none have been extensively evaluated, leaving their performance in a clinical setting largely unknown.

Enterovirus and Parechovirus

Enterovirus and parechovirus are positive-sense single-stranded RNA viruses belonging to the family *Picornaviridae*. There are 63 distinct members of the genus *Enterovirus* and four recognized members of the genus *Parechovirus*. Enteroviruses and human parechoviruses (HPEVs) are ubiquitous agents found worldwide (117, 124, 223, 246). In temperate climates, the majority of enterovirus and parechovirus infections occur in late summer and fall, with infections occurring into the winter. It has been estimated that enteroviruses infect 1 billion or more individuals worldwide each year, and in the United States, it is estimated that between 30 and 50 million infections occur annually. Several modes of transmission exist for these viruses, including fecal-oral, respiratory, transplacental, perinatal, and self-inoculation modes, but the majority are fecal-oral (115, 185). The highest incidence of enterovirus infection is in infants and young children, while infants under the age of 12 months have the highest incidence of parechovirus infection (20, 21, 24). The majority of enterovirus infections are asymptomatic (40). Children often have respiratory symptoms, and this syndrome has been referred to as a "summer cold." Historically, the most serious diseases due to enteroviruses have been poliomyelitis and aseptic meningitis. Poliovirus is no longer present in North America, but enterovirus continues to be the most common cause of aseptic meningitis (116). Other acute enterovirus syndromes include hemorrhagic conjunctivitis, hand-foot-and-mouth disease, herpangina, Borhnholm disease, and pleurodynia (40). Symptomatic infections due to parechoviruses are primarily respiratory (colds and pneumonia) or gastrointestinal (21, 247); however, acute flaccid paralysis and severe encephalitis has been associated with HPEV (83, 141). A third HPEV and a fourth HPEV were recently discovered in Japan and The Netherlands, respectively (20, 120).

Enterovirus can be detected using a variety of methods including culture, SVC, and NAATs. Although enteroviruses grow well in a variety of cell lines, no one cell line can detect all serotypes, limiting the sensitivity of culture unless three or four cell lines are used (43). Other disadvantages of culture are that it can take up to 8 days for CPE to appear when virus is present in low titers (for example, in cerebrospinal fluid [CSF] specimens), and some type A coxsackieviruses do not grow in cell culture (223). The time required to isolate enterovirus in traditional culture is too long to be of any use clinically. Although SVC using monoclonal antibodies has decreased the culture time compared with that for tube culture, it is less sensitive than conventional culture (136). The use of commercial Super E-Mix cells, a mixture of BGMK and A549 cells (Diagnostic Hybrids Inc., Athens, OH), in SVC has improved the sensitivity of detection, and in one study, SVC was slightly more sensitive than conventional culture (31). A variety of cells including tertiary monkey kidney cells, embryonic lung cells, African green monkey kidney cells, HT29 cells, LLC-MK2 cells, and A549 cells have been used to isolate HPEV, but comparisons have not yet been reported (1, 247).

The poor yield of culture plus the fact that some coxsackieviruses fail to grow in culture are the reasons that laboratories have turned to molecular methods for detecting enterovirus. RT-PCR and NASBA assays have been used to detect enterovirus and parechovirus (Table 9). Most enterovirus PCR assays target the conserved 5′-NCR of the genome, and both consensus and serotype-specific assays have been described (224, 225). As mentioned above, some primers in the 5′-NCR also detect rhinovirus, and if enterovirus needs to be distinguished from rhinovirus, a second PCR targeting the VP1 or VP4 gene is required (32, 123, 148). Rotbart described the first RT-PCR for enterovirus; this assay used primers to amplify the 5′-NCR and was more sensitive than culture (226). In comparisons between RT-PCR and cell culture, the sensitivity of RT-PCR for specimens with low virus titers (CSF specimens) has been both higher and lower than cell culture and may reflect how well different laboratories culture enterovirus (226). The NucliSens Easy Q enterovirus kit is a NASBA test for enterovirus detection (232). The sensitivities of RT-PCR and NASBA are similar and exceed that of SVC for enterovirus detection (31, 145). NASBA has been evaluated in several laboratories by comparing NASBA to culture and RT-PCR. Capaul and Gorgievski-Hrisoho (32) tested 141 specimens, and NASBA detected 45 positive specimens, compared to 50 for RT-PCR. Landry et al. compared NASBA to a commercial RT-PCR (Argene Enterovirus Consensus RT-PCR) and culture; NASBA was slightly more sensitive than RT-PCR, detecting 39 of 44 positive specimens, compared with 37 of 44 for RT-PCR (153). Both NAATs were significantly more sensitive

TABLE 9. Molecular tests for detection of enterovirus and parechovirus[a]

Assay format	Gene target(s)	Sensitivity (%)	Specificity (%)	Description	Reference
RT-PCR	5′-NCR (409 bp)	ND	ND	First reported case of HPEV1 associated with encephalomyelitis	156
RT-PCR	5′-NCR (154 bp)	ND	ND	RT-PCR was more sensitive than culture for enterovirus detection	226
Real-time RT-PCR	5′-NCR	ND		One-step and two-step RT-PCR assays had similar sensitivities based on 24 positive specimens; LLOD of 510 GE/ml CSF; enterovirus-specific TaqMan probe distinguished enterovirus from rhinovirus	148
NASBA RT-PCR	5′-NCR	ND	ND	NucliSens Easy Q enterovirus kit and in-house RT-PCR were compared using 141 specimens; NASBA detected 45 positive specimens, compared to 50 for RT-PCR	32
Real-time RT-PCR	5′-NCR	100	100	LightCycler real-time and end-point RT-PCR both detected 38/38 positive specimens, while culture detected 12/32 (37.5%)	130
NASBA RT-PCR	Not given	92.9 (NASBA), 88.1 (RT-PCR)	100 (NASBA), 94.7 (RT-PCR)	NucliSens Base kit (NASBA) and Argene Biosoft enterovirus consensus RT-PCR detected 39/44 and 37/44 positive specimens, respectively, compared with only 23 for culture	153
Multiplex RT-PCR	5′-NCR EV (116 bp), HPEV (253 bp)	ND	ND	Multiplex RT-PCR for detection of enterovirus, rhinovirus, and HPEV had an analytical sensitivity of 100 GE; was equally as sensitive as traditional methods	123
RT-PCR	VP1 (972 bp)	ND	ND	RT-PCR detected HPEV types 1, 2, and 3 but not enterovirus	1
RT-PCR	Near VP1 (760 bp)	ND	ND	RT-PCR detected 37 of 284 culture-positive specimens for enterovirus	20
RT-PCR	5′-NCR	97.1	100	The GeneXpert EV kit was compared to in-house RT-PCR and SmartCycler ASR (Cepheid) using 102 CSF specimens and 34 positive specimens	142
Multiplex RT-PCR	Proprietary	ND	ND	ResPlex II detects 12 viruses; detected 19 (5.3%) enterovirus-positive specimens out of 360 frozen specimens	161

[a] ND, not determined; VP1, viral capsid protein 1.

than culture, which detected only 23 positive specimens. The NASBA enterovirus test does not cross-react with rhinovirus and therefore does not require a second amplification to distinguish them. Currently, the Xpert EV test from Cepheid (Sunnyvale, CA) is the only FDA-approved test for enterovirus detection (232). Two recent evaluations of this test have appeared. In a multicenter evaluation including 102 CSF specimens and 34 confirmed positive specimens, the Xpert EV assay had a sensitivity of 97.1% and a specificity of 100% (142). In a second unblinded study of 83 selected positive specimens and 79 selected negative specimens, the Xpert EV test had a sensitivity of 98.8%, compared with 97.6% for the Argene enterovirus consensus RT-PCR and 100% for an in-house assay (232). The xTAG RVP assay is FDA approved for picornavirus detection and detects both rhinovirus and enterovirus without distinguishing between the two. It has a sensitivity and a specificity of 100% and 91.3%, respectively, for picornavirus (34). A number of ASRs, including the Enterovirus Blend from Chemicon Light Diagnostics (Temecula, CA), the MGB Alert real-time PCR enterovirus assay from Nanogen (San Diego, CA), or NASBA (EasyQ enterovirus reagents; bioMerieux, Durham, NC), are commercially available. HPEVs have been detected by RT-PCR targeting the 5′-NCR and the N-terminal VP1 regions (1, 20, 21), and a few studies have shown that RT-PCR for parechovirus detection is more sensitive than culture (130, 156, 290). There are no commercially available tests specifically designed to detect HPEV. It is not clear whether the GeneXpert enterovirus kit or the xTAG RVP assay will detect HPEVs.

hMPV

hMPV is an RNA virus that was discovered in The Netherlands in 2001 (279). Together with RSV, hMPV belongs to the family *Paramyxoviridae*, the subfamily *Pneumovirinae*, and the genus *Pneumovirus* (278). Mackay et al. (169) sequenced the P, M, and N genes and showed that hMPV can be divided into two main lineages (A and B), each with two sublineages (A1, A2, B1, and B2). Schildgen et al. (231) reported a new variant of hMPV based on N gene sequence data that may represent

a fifth genotype; however, this genotype has not been confirmed by other investigators. hMPV causes both URTI and LRTI, and the signs and symptoms are very similar to those caused by RSV, ranging from mild rhinorrhea associated with common colds to severe cough, wheezing, bronchiolitis, and pneumonia (139, 280). hMPV outbreaks occur predominantly in the winter and spring months in temperate climates, often overlapping or following the winter RSV outbreak (127, 222, 281). In studies where the detection of hMPV across several seasons was compared, investigators found significant differences from year to year. In studies where genotyping was performed, the predominant genotype changed from one year to the next (242, 287). Some studies, however, have shown that sporadic hMPV infections can occur year round (301). Seroprevalence studies conducted in The Netherlands indicate that most of the population is infected by the age of 5 years; however, infections occur in all age groups (25, 209, 252). The incubation period is thought to be 3 to 5 days, and the period of viral shedding has not been determined but may be weeks following primary infection in infants (257, 289). Transmission is believed to occur by contact with respiratory secretions involving large-particle aerosols, droplets, or contaminated surfaces, and nosocomial infections have been reported. hMPV is often the second or third most prevalent virus detected, and in hospitalized children with ARD under the age of 5 years (193), the prevalence has ranged from 3 to 25% (Table 2). The overall positivity rate when the results from 18 studies in 12 countries were combined was 6.9% (602/8,681). This compares well with the prevalence of hMPV in the largest study, reported by Sloots et al. (242), who examined 10,025 specimens collected from 2001 to 2004 and found an annual positivity rate of 7.1%. Although a significant number of patients with exacerbations of asthma have hMPV infection, it is not clear what role hMPV plays in long-term wheezing. Recent publications employing NAAT for several viruses indicated that hMPV is often present in dual infections, which occur at significant frequencies, ranging from 4.8 to 30% (45, 93, 177, 211).

hMPV infections can be diagnosed using a variety of approaches including serology, virus isolation, and antigen or nucleic acid detection. The virus grows best in tertiary monkey kidney or LLC-MK2 cells and produces a moderate CPE consisting of small, round, granular, and refractile cells without large syncytium (25). The major drawback of cell culture, however, is the length of time required to identify virus and its poor sensitivity. In a comparison of PCR and culture involving 637 specimens, Ebihara et al. (64) detected 57 of 637 (8.9%) hMPV-positive specimens by RT-PCR, compared with only 7 of 268 (2.6%) specimens detected by culture. The recent development of monoclonal antibodies by Italian workers and the CDC has facilitated the detection of hMPV in nasopharyngeal swabs by DFA (208). In one study from Italy, DFA had a sensitivity of 73.9% and a specificity of 94.1% compared to RT-PCR (208). Landry et al. (152) used a commercially available monoclonal antibody (MAB8510, prepared by the CDC and available through Chemicon) to evaluate A549, HEp-2, and LLC-MK2 cells in SVC and found that all three cell lines detected hMPV equally on day 2. An SVC method using R-Mix cells (Diagnostic Hybrids Inc., Athens, OH) and a specific monoclonal antibody has recently been introduced for hMPV detection. In one study, the sensitivity of SVC was 100% compared with tube culture (217). An ELISA to measure hMPV antibody has been described; however, antibody detection did not correlate well with RT-PCR results, limiting the usefulness of serology for diagnosing infections (77).

Molecular tests are the most sensitive method for hMPV detection, and several NAATs employing a variety of amplification targets including the polymerase (L), matrix (M), fusion (F), or nucleoprotein (N) genes have been described (Table 10). Van den Hoogen et al. (280) described the first RT-PCR for hMPV detection. This PCR amplified a 171-bp fragment of the polymerase (L) gene. Boivin et al. (25) described RT-PCR assays that amplify the F gene (759 bp) and M gene (780 bp) for identifying 38 isolates of hMPV. The following year, MacKay et al. (170) described a plate hybridization assay and a real-time LightCycler assay using N gene primers. The real-time assay had good reproducibility, with a small intra-assay coefficient of variation. Côté et al. (51) developed PCR assays for five gene targets and showed that the N and L gene assays were more sensitive than the M, P, and F gene assays using 20 positive specimens. Greensill et al. (100) used an M gene assay (120 bp) as a screening assay, followed by confirmatory assays targeting the F (134 bp), N (325 bp), and M (331 bp) genes. Well-optimized NAATs have been able to detect 0.01 $TCID_{50}$ of hMPV (29, 230). A NASBA assay for hMPV that appears to be slightly less sensitive than RT-PCR has been described. NASBA had an LLOD of 100 copies, compared to 50 copies for RT-PCR (54). In the absence of published studies with significant numbers of specimens tested, it is not clear how well NASBA performs. Surprisingly, 5 years after the first reports of hMPV, there are few good comparisons of the sensitivities or performances of different PCR assays. Maertzdorf et al. (172) developed a real-time assay for the N gene and showed that the real-time assay was 10-fold more sensitive than the original L gene (171 bp) assay and was able to detect all four genotypes with equal sensitivities. Dare et al. (54) also showed that all four genotypes could be detected by real-time RT-PCR targeting the N gene. Quantitative real-time PCR assays have also been used to measure viral loads present in respiratory specimens. Kuypers et al. (147) showed that hMPV viral loads ranged from 4.4×10^4 to 6.7×10^7 copies per ml. Scheltinga et al. (230) described a multiplex assay to detect hMPV and rhinovirus with an LLOD of 0.01 $TCID_{50}$ for hMPV, which was more sensitive than culture based on testing of only six positive cultures.

Commercial products have recently been introduced for hMPV detection. ASR reagents including monoclonal antibodies and probes and primers for PCR are available from several companies. DFA reagents are available in the Diagnostic Hybrids D^3 Ultra 8 DFA respiratory virus detection, MPV-specific DFA reagent (Diagnostic Hybrids Inc., Athens, OH), Imagen hMPV DFA test (Thermo Fisher Scientific, Ely, United Kingdom), and Chemicon SimulFluor hMPV DFA reagent (Millipore Corporation) kits. Available probes and primers for PCR include the NucliSens hMPV primer-probe mix (bioMerieux, Durham, NC), the Nanogen RVA ASR (Nanogen Inc., San Diego, CA), the Pro hMPV+ Real Time Assay RUO (Prodesse Inc.), the Qiagen RUO ResPlex II panel (Qiagen, Hamburg, GmbH), and the Seeplex RV detection kit (Seegene Inc., Seoul, South Korea). hMPV has also been detected using commercial multiplex assays capable of detecting

TABLE 10. Molecular tests for detection of hMPV[a]

Assay format	Gene target(s)	Sensitivity (%)	Specificity (%)	Description	Reference(s)
RT-PCR	F (450 bp), N (377 bp)	ND	ND	RT-PCR detected 11 isolates identified by cell culture; no performance data	209
RT-PCR	F (759 bp), M (780 bp)	ND	ND	RT-PCR detected 38 isolates that grew in cell culture	25
RT-PCR	L (170 bp)	ND	ND	RT-PCR detected 52 positive specimens out of 1,515 specimens	280
Real-time RT-PCR	N (928 bp), L (549 bp), M (778 bp), P (876 bp), F (758 bp)	ND	ND	Real-time LightCycler RT-PCR assay using 5 gene targets; N gene assay was most sensitive; N gene assay had an LLOD of 100 copies, with sensitivity equivalent to culture	51
Real-time RT-PCR	N	ND	ND	Real-time RT-PCR LightCycler assay was more sensitive than end-point assays using either agarose gel detection or ELISA microtiter plate detection	170
RT-PCR	M (120 bp), F (134 bp), N (325 bp)	ND	ND	M gene RT-PCR-positive specimens confirmed using F and N gene assays	100
Real-time RT-PCR	N (199 bp), F (450 bp)	ND	ND	N gene RT-PCR detected 6/94 positive specimens; F gene amplification identified no genotype	29
Multiplex RT-PCR	L (170 bp)	ND	ND	Multiplex RT-PCR detected hMPV and rhinovirus; LLOD of 0.01 $TCID_{50}$ for hMPV; RT-PCR more sensitive than culture based on only 6 positive specimens	230
RT-PCR	P (325 bp), M (184 bp), N (262 bp)			P gene RT-PCR assay followed by sequencing amplification identified all four genotypes	169
Nested RT-PCR	N (320 bp)	ND	ND	N gene RT-PCR amplification and sequencing identified a fifth genotype	231
Real-time RT-PCR	N, L (170 bp)	ND	ND	N gene real-time LightCycler RT-PCR assay detected all known lineages of hMPV; LLOD of 0.01 $TCID_{50}$; more sensitive than L gene end-point RT-PCR assay	172
Real-time RT-PCR NASBA	NASBA proprietary N (RT-PCR)	ND	ND	Real-time NASBA (bioMerieux) had an LLOD of 100 copies; real-time RT-PCR had an LLOD of 50 copes; RT-PCR was more sensitive than NASBA	54
Real-time RT-PCR	F (70 bp)	ND	ND	Real-time RT-PR detected 1,000 copes of all four hMPV lineages; assay quantitative from 10 to 10^8 copies	147
Multiplex RT-PCR	N	96.0	98.8	Multiplex RT-PCR detects 19 respiratory viruses; LLOD of 0.1 $TCID_{50}$/ml; RT-PCR more sensitive than DFA + culture	34, 143, 176
Multiplex RT-PCR	Proprietary	80	99.7	ResPlex II detects 12 viruses compared to culture and TaqMan PCR using 360 frozen specimens; detected 13 positive specimens	161
Multiplex RT-PCR	Proprietary	ND	ND	MultiCode-PLx detects 17 viruses using the Luminex xMAP system; tested 354 specimens by DFA and culture; detected 9 positive specimens, all confirmed by a second PCR	200

[a] ND, not determined; F, fusion protein; N, nucleoprotein; M, matrix protein; P, phosphoprotein; L, large polymerase subunit.

several respiratory viruses. These assays include the ResPlex II (Qiagen), the MultiCode-PLx respiratory virus panel (EraGen Biosciences), the Nanogen RVA ASR (Nanogen), and the xTAG RVP assay (Luminex Molecular Diagnostics). The sensitivity and specificity of the ResPlex II and MultiCode-PLx assays for detecting hMPV have not been determined (200). The xTAG RVP assay is the only FDA-approved molecular test for hMPV and has a sensitivity of 96% and a specificity of 98.6% (34).

CoV

All CoVs belong to the genus *Coronavirus* within the family *Coronaviridae* and are positive-sense single-stranded RNA vi-

ruses with a genome of 27 to 32 kb, the largest of all RNA viruses. There are five HCoVs, which include 229E, OC43, SARS-CoV, NL63, and HKU1. HCoV-229E and -NL63 are group I CoVs, and OC43, SARS-CoV, and HKU1 are group II CoVs. HCoV-OC43 and HCoV-229E were identified in the mid-1960s as being a cause of mild self-limited URTI and were subsequently shown to cause about one-third of "common cold"-like illnesses in adults. Like other respiratory viruses, HCoV-OC43 and -229E are spread by large droplet infection. Overall, they account for 5 to 30% of respiratory tract infections, and outbreaks may occur in 3- to 4-year intervals (277). HCoV-OC43 and -229E infections can present with a variety of signs and symptoms ranging from a self-limiting common cold including cough, runny nose, and fever to bronchiolitis or pneumonia. HCoV-OC43 and -229E have been associated with both URTI and LRTI in a variety of settings including nosocomial infections in high-risk immunocompromised children, in hospitalized elderly patients with non-influenza-virus ARD and pneumonia, and in newborns, children, and hospital staff (79, 207, 237, 273). Of concern to infection control practitioners is the recent finding that HCoV-229E can survive for up to 3 h when dried on solid surfaces and for up to 6 days in saline solution at room temperature.

The third HCoV, NL63, was discovered in The Netherlands in a 7-month-old boy who presented with coryza, conjunctivitis, and fever who had chest X-ray findings consistent with bronchiolitis (282). The virus grew in tertiary monkey kidney cells, which distinguished it from HCoV-OC43 and -229E. Sequencing of the genome indicated that it was not a recombinant virus but genetically distinct from all known HCoVs. Following the first case, NL63 was detected in five additional children and three adults, two of whom were immunocompromised. A second group in The Netherlands independently reported a novel group 1 CoV with a 27,555-nucleotide genome with 34% GC content (86). This virus, from an 8-month-old with pneumonia, was first isolated in 1988 in monkey kidney cells and is most likely the same virus as NL63. Using an RT-PCR assay and primers based on the first isolate, they were able to detect this virus in 4 of 139 specimens collected from pediatric patients with URTI between 2000 and 2002, indicating that this virus has been circulating in The Netherlands for a number of years. Almost a year later, a third group in New Haven, CT, described a novel group I CoV, which they called HCoV-NH, that turned out to the same virus (72). Since the initial reports from The Netherlands, NL63 has been found worldwide (Table 2). NL63 infections occur predominantly in temperate climates in winter months, with a peak in November (106); infections have occurred in spring or summer months only in Hong Kong. This suggests that the seasonality of NL63 infections may be different in tropical and subtropical climates. In the majority studies, the highest proportion of infections were in children <5 years of age. Analysis of the clinical findings from several studies indicates that NL63 usually causes mild respiratory tract illness, although in some studies, there was an association with croup. More recent studies have established a strong link between NL63 infection and croup. In one German study, almost half of the patients with NL63 had croup, and a higher proportion of specimens from patients with croup yielded NL63 than specimens from patients without croup (85). The association with croup was not seen in some studies, possibly because of patient selection bias (283). NL63 is often present as a coinfection with another virus, and in one study, the frequency of double infections exceeded 50% (41, 283). Dual infections have been observed in hospitalized patients more often than in outpatients (283), suggesting that patients with a dual infection may have more severe disease or a longer course of infection than patients with a single NL63 infection. NL63 infection has recently been associated with Kawasaki disease (70), but this association has not been confirmed in three follow-up studies in Japan and Taiwan (65). As is the case for other CoVs, the period of viral shedding for NL63 may be protracted. The virus is still present in respiratory specimens 3 weeks after the initial infection in 50% of infected children (128). This observation is particularly important for infection control practitioners, given the increased opportunity for nosocomial infections.

The fifth HCoV, HKU1, was discovered in January 2004 in a 71-year-old man returning to Hong Kong from Shenzhen, China (303). He presented with fever and a productive cough with purulent sputum and had a chest radiograph showing patchy infiltrates. All attempts to grow a virus failed, but CoV RNA was detected in the NPA by RT-PCR using *pol* gene consensus primers. Quantitative PCR indicated high titers of virus in the NPA during the first week of illness with decreasing titers in the second week and undetectable levels of virus in the third and fourth weeks. A second Hong Kong case was subsequently identified, a 35-year-old women with pneumonia with unknown etiology. The following year, a report from Brisbane, Australia, described the detection of HKU1 in 10 children with respiratory tract infections during the winter (241). Three different HKU1 genotypes (genotypes A, B, and C) have now been identified, and genotypes A and B have been more prevalent than genotype C in studies for Italy and Australia (96, 241). Following these initial publications, there have been 10 reports from eight countries in four continents describing the detection of HKU1 in children and adults worldwide (Table 2). In Brisbane, children under the age of 2 years were most susceptible to HKU1 infection. In the New Haven study, the most common presenting signs were rhinorrhea, cough, fever, and abdominal breath sounds on auscultation; hypoxia was present in only one child, and four had abnormal chest radiographs (71). Pneumonia, bronchiolitis, and acute asthma exacerbations were seen in children with HKU1 infections in Hong Kong. In the study from Seattle, infections occurred in every month except July, with a peak in December 2003. In a 2-year study in Hamilton, Canada, infections occurred in only 1 year, suggesting annual variation in HKU1 infections (177).

Before the advent of molecular tests for respiratory viruses, CoV infections were diagnosed by serology or virus isolation. Culture has proven to be unreliable for the primary isolation of CoVs as some strains grow poorly in cell culture (175), and CPE often takes 7 to 10 days to develop. CoV antigens have been detected in cell culture inoculated with nasopharyngeal or throat wash specimens by standard IF staining with CoV-specific monoclonal antibodies (239), and SARS-CoV antigens have been detected by ELISA (163), but this method has not yet been reported for other CoVs. Serological assays have not been used to diagnose infection, but these assays have been used only in epidemiological studies. HKU1 antigens have been detected by DFA in cells from the nasopharynx or throat washes by using a newly developed group-specific monoclonal

antibody (96). DFA, however, was less sensitive than RT-PCR, detecting only 8 of 10 RT-PCR-positive specimens (96).

A variety of molecular tests have been used for detecting CoV including RT-PCR, NASBA, and LAMP amplification techniques (Table 11). For RT-PCR, conventional heat block or real-time assays using one-step or two-step formats have been used to detect CoVs using consensus primers that amplify the *pol* 1b or nucleocapsid gene (2, 303). For a two-step assay, cDNA is prepared using random hexamer primers, and the cDNA is used as an input for PCR amplification. Most two-step assays can be readily converted to a one-step RT-PCR without a loss of sensitivity. CoVs have been detected by using both consensus and CoV-specific primers. Two recent reports have described consensus *pol* 1b gene primers for the detection of CoV RNA. Adachi et al. (2) used consensus primers to amplify a 220-bp fragment of *pol* 1b with an LLOD of 10 GE in a one-step assay. They tested 44 SARS-CoV specimens, CoV-229E and -OC43, and turkey infectious bronchitis virus and showed that this consensus RT-PCR assay could detect all four CoVs. Woo et al. (303) described a two-step conventional heat block assay that used consensus primers to amplify a 440-bp fragment of the *pol* 1b gene (304). This assay was used to detect a novel human respiratory CoV, HKU1 (see section below), in a 71-year-old patient with non-SARS pneumonia during the SARS outbreak in Hong Kong.

CoV-NL63 has been detected using both consensus and NL63-specific primers. van der Hoek et al. (282) used two nested PCR assays, one targeting the 1b gene (237-bp product) and one targeting the 1a gene (525-bp product); the former was used as a screening PCR, and the latter was used as a confirmatory PCR. Both RT-PCR assays were one-step reactions (combined RT and PCR), and in each case, 1 μl of the first-round product was put into the nested PCR. This two-nested-PCR assay approach has been used by other investigators (9, 106), but nested assays have given way to nonnested assays. Fouchier et al. (86) optimized a nonnested PCR assay by using three sets of primers and TaqMan probes directed at the nucleocapsid gene and showed that one set of primers was superior to the other two in analytical sensitivity. Real-time assays using either a LightCycler (Roche Diagnostics, Basel, Switzerland) or ABI 7500/7700/7900 (Applied Biosystems Inc., Foster City, CA) instrument have been developed using either Sybr green, a minor groove dye, TaqMan probes, or fluorescence resonance energy transfer probes (71). Several investigators have used a combination of assays that target the *nuc* and *pol* 1a genes to confirm positive results or to type CoVs (16, 17). Moës et al. (190) used a pan-CoV PCR targeting a 251-bp fragment of the *pol* gene, followed by three NL63-specific assays targeting the nucleocapsid gene (314-bp product), the 1b gene (237-bp product), or the spike gene (663-bp product). Esper et al. (72) used a pan-CoV set of primers (550-bp product) for screening and a second NL63-specific confirmatory PCR targeting the 1a gene (215-bp product). The pan-CoV PCR was less sensitive than the NL63-specific PCR assay for detecting NL63 in clinical specimens. Those authors suggested that this was likely due to the many mismatches that they observed by sequencing through the primer binding regions of the NL63 isolates. The analytical sensitivities of the assays were not determined.

HKU1 has been detected by RT-PCR using both consensus and HKU1-specific PCR assays (Table 11). Woo et al. (303) described a two-step conventional heat block assay that used consensus *pol* 1b primers that amplify a 439-bp fragment of the HKU1 *pol* 1b gene. Adachi et al. (2) described a second consensus *pol* 1b PCR that amplifies a 220-bp gene fragment in a one-step assay with an analytical sensitivity of 10 GE. Another set of pan-CoV primers described by Moës et al. (190) targets the *pol* 1ab gene. One CoV consensus assay that uses six primers and two TaqMan probes for screening, followed by reflexing into assays using type-specific primers using individual primers from the pooled primer set, has been described (146). A number of HKU1-specific PCR assays that amplify the polymerase, spike, and nucleocapsid genes have been developed. A 453-bp fragment of the *pol* gene first developed by Woo et al. (303) has been used widely in several studies. Garbino et al. (92) used real-time PCR assays targeting the polymerase gene for OC43 and 229E and the replicase gene of NL63 run on an ABI 7900 HT for 55 cycles. Esposito et al. (73) used individual real-time PCR assays for 229E, OC43, NL63, and HKU1 with amplicon sizes ranging from 64 to 98 bp. One report used an HKU1-specific S1 gene PCR to generate a 713-bp fragment (274). The PCR conditions for most of these studies are poorly described, and amplification cycles have ranged from 40 to 55 cycles. In some cases, analytical sensitivity is provided, but in many cases, it is not, and little assay validation data are provided. A somewhat unique approach using a consensus RT-PCR followed by a low-density oligonucleotide array to differentiate different CoV types was recently reported (58). This one-step RT-PCR uses two sense primers and three antisense primers to amplify the polymerase gene in a 50-cycle touchdown PCR. Amplification products are hybridized to type-specific oligonucleotides immobilized on plastic, and results are read with the naked eye. The LLOD was reported at 100 copies, similar to that obtained with a gel and individual virus-specific real-time RT-PCR based on testing a sample of 39 specimens (58). A multiplex RT-PCR assay utilizing a fluidic microbead array and the Luminex xMap system for the detection of 19 respiratory viruses including five CoVs, 229E, OC43, SARS, NL63, and HKU1, has been described (176). This assay, the xTAG RVP assay (Luminex Molecular Diagnostics, Toronto, Ontario, Canada), has not been FDA cleared for CoV detection. The xTAG RVP assay has a lower limit of detection of 50 GE for each CoV and has been used to study the epidemiology of CoV infections across multiple seasons (177). Another multiplex PCR assay that also uses the Luminex xMap system, MultiCode-PLx from EraGen Biosciences (Madison, WI), detects 17 different respiratory viruses but does not include HKU1 CoV (138, 188).

Bocavirus

Human bocavirus (HBoV) is a single-stranded DNA virus within the family *Parvoviridae*, subfamily *Parvovirinae*, and genus *Bocavirus*. HBoV was discovered in September 2005 by Allander and colleagues in Sweden while testing pooled nasopharyngeal specimens using large-scale molecular viral screening techniques including DNase sequence-independent single-primer amplification (4). In the original study, 540 nasopharyngeal specimens obtained from hospitalized patients were tested for HBoV, and 3.1% were positive. There

TABLE 11. Molecular tests for detection of CoV[a]

Assay format and organism	Gene target(s)	Sensitivity (%)	Specificity (%)	Description	Reference(s)
Pan-CoV					
RT-PCR	*pol* 1b (220 bp)	ND	ND	RT-PCR amplifies consensus region of *pol* 1b genes of five CoVs; LLOD of 10 GE; CoV type identified by RE digestion or amplicon sequencing	2
RT-PCR	*pol* 1b (440 bp)	ND	ND	RT-PCR amplifies consensus region of *pol* gene; used to detect first cases of HKU1	303
Multiplex RT-PCR	*pol*	ND	ND	xTAG RVP assay detects 17 respiratory viruses including 5 CoV types (229E, OC43, NL63, HKU1, and SARS CoV) using microbead array and the Luminex xMAP system	176
NL63		ND	ND		
Nested RT-PCR	*pol* 1b (237 bp), *pol* 1a (525 bp)	ND	ND	Nested RT-PCR followed by sequence used for discovery of NL63; no performance data	282
Nested RT-PCR	*pol* 1a (525 bp), *pol* 1b (237 bp)	ND	ND	Second nested RT-PCR used to confirm positive results	9, 106
RT-PCR	*pol* 1a (251 bp), *pol* 1b (314 bp), *pol* 1b (237 bp), S (663 bp)	ND	ND	Pan-CoV RT-PCR detects all 5 CoVs	190
HKU1		ND	ND		
RT-PCR	*pol* 1b (439 bp)	ND	ND	RT-PCR detected 9 of 1,048 specimens as being HKU1 positive; no assay performance data	67
RT-PCR	*pol* 1b (440 bp), 229E (294 bp), OC43 (469 bp)	ND	ND	RT-PCR using consensus and type-specific primers	41
Real-time RT-PCR	N (64–98 bp)	ND	ND	RT-PCR detected 79 CoV-positive specimens out of 2,060 children tested; no performance data provided	73
RT-PCR	HKU1 N (443 bp), S (713 bp)	ND	ND	Type-specific RT-PCR assays for 4 CoVs detected 10 positive specimens out of 135 specimens tested; no performance data	274
Multiplex RT-PCR	Proprietary	ND	ND	Multiplex RT-PCR (MultiCode-PLx; EraGen) followed by fluidic microbead array used to detect 17 respiratory viruses including 229E, OC43, and NL63; more sensitive than DFA and culture	138, 188
RT-PCR	*pol* 1b (85–100 bp)	ND	ND	Consensus and type-specific RT-PCR assays used 4 primer pairs and 3 TaqMan probes; LLOD of 10 copies; detected 66 CoV-positive specimens out of 1,043 specimens tested	146
RT-PCR	*pol*	100	ND	Consensus RT-PCR followed by low-density array for CoV identification was as sensitive as individual RT-PCR assays, detecting 39/39 positive specimens; LLOD was 100 transcripts	58
RT-PCR	*pol* (453 bp)	ND	ND	RT-PCR detected 10 out of 418 positive specimens; no confirmation; no performance of assay	304
Nested RT-PCR	229E N (47 bp), OC43 N (496 bp)	ND	ND	Type-specific RT-PCR assays for 229E and OC43 detected 28/261 positive specimens for patients with RT1 and 1/243 without RT1	285
RT-PCR	*pol*	ND	ND	Type-specific RT-PCR assays identified 87 CoV-positive out of 4,181 specimens tested; no performance data	155
Multiplex RT-PCR	Proprietary	ND	ND	MultiCode-PLx detected 17 viruses including 229E, OC43, and NL63; 354 specimens tested by DFA and culture; detected 3 positive specimens, and all 3 were confirmed by a second PCR	200

[a] ND, not determined. RE, restriction endonuclease; *pol*, polymerase gene; N, nucleocapsid protein; S, spike protein.

appears to be two genotypes of HBoV circulating worldwide. Genotypes ST1 and ST2 differ by only 26 nucleotides across the entire genome, with most of the conserved amino acid changes located in the VP1/VP2 open reading frame (4, 134, 195). Smuts and Hardie (244) were able to demonstrate two sublineages (three nucleotide changes) of the ST2 lineage by sequencing the 980-nucleotide VP1/2 gene. All of the specimens were from young children with respiratory distress, mainly acute wheezing, and many had pneumonia with interstitial infiltrates seen by chest radiography. Since that first publication, there have been numerous studies reporting HBoV prevalences ranging from 2 to 11% in respiratory tract specimens from over 12 countries on five continents including Europe, North America, Asia, and Australia, indicating that this virus is distributed worldwide (Table 2). In most studies where the frequencies of several viruses were determined, HBoV was most prevalent in children <3 years of age and less common than RSV and rhinovirus; approximately as common as influenza virus, hMPV, PIV3, and adenoviruses; and probably more common than CoVs and other PIVs. HBoV infections occur in both children and adults, but children under the age of 2 years appear to be most at risk for infection. HBoV has been detected in children with LRTI and has been associated with abnormal chest radiographic findings in several studies, but the causative role was not clearly established. In these studies, HBoV was detected concurrently with other viral pathogens in one- to two-thirds of cases, where the question of whether HBoV was causing the respiratory illness was raised (183). In a recent study by Allander et al. (3), HBoV was detected in 49 (19%) out of 259 children hospitalized with acute wheezing, and 12 of these children had only HBoV detected in nasopharyngeal specimens (3). High viral loads were detected in most children with acute wheezing when HBoV was the only virus detected. HBoV was also detected in serum specimens of patients with acute wheezing. This, coupled with two recent studies indicating that HBoV is rarely detected in asymptomatic individuals (134, 178), is strong evidence that HBoV can cause LRTI, in particular, acute wheezing. In studies from five countries, HBoV infections have occurred from October to April with a peak in December and January, with few infections in summer months (4, 10, 42, 134, 178).

HBoV infections have been diagnosed almost exclusively using molecular methods, and HBoV has not yet been isolated in cell culture. The genomic organization of HBoV closely resembles that of other bocaviruses with open reading frames for a nonstructural gene, NS-1, followed by an unknown protein, NP-1, and followed by two capsid protein genes, VP1 and VP2 (134). Several PCR assays have been described for HBoV, and most have amplified NS-1 and NP-1 genes (Table 12). The first PCR described by Allander et al. (4) was a conventional heat block assay that targeted the N terminus of the NP-1 gene, yielding a 354-bp product. Smuts and Hardie (244) used two seminested PCR assays targeting a 368-bp region of the NP-1 gene and a 980-bp fragment of the VP1/VP2 capsid gene; the former was used as a confirmatory test. Other investigators targeted the NS-1 gene using either a nonnested PCR producing a 291-bp fragment (173, 241) or a nested PCR (178). Manning et al. were able to confirm 53 of 54 positive specimens using a nested PCR (178). Some investigators used two PCR assays, one as a screening assay and one as a confirmatory assay. In most studies where NS-1 and NP-1 targets are used, the majority of positive specimens were confirmed using a second PCR, indicating that these assays have similar sensitivities (167). Allander et al. (3) described a real-time assay that was more sensitive than an end-point assay (LLOD of 10 GE), detecting 33% more positive specimens. Two real-time PCR assays targeting the NS-1 and NP-1 genes of HBoV were described by Lu et al. (167), and both assays had similar sensitivities, with an LLOD of 10 GE. Eighteen of 71 positive specimens could not be confirmed by a second PCR, raising a question about the specificity of this assay. Arnold et al. (10) described a real-time assay targeting the NP-1 gene with an LLOD of 17 copies. This assay detected 182 positive specimens out of 1,474 specimens and had good specificity, as all positive results were confirmed by a second assay targeting the NS-1 gene. In our hands, a real-time LightCycler assay targeting the NP-1 gene had sensitivities similar to those of an end-point assay targeting either NS-1 or NP-1 (177). At the time of writing, there were no FDA-approved tests for HBoV; however, the second version of the xTAG RVP test, currently an RUO test, detects HBoV.

Parvovirus Types 4 and 5 and Mimivirus

Human parvovirus type 4 was first identified in 2005 in the plasma of a patient with acute viral syndrome following high-risk behavior for HIV-1 transmission (125). Parvovirus type 4 and a similar virus, parvovirus type 5, have been detected in serum or plasma specimens of 70% of HIV-infected patients (179), in about 4% of manufactured plasma pools, and in plasma from healthy blood donors (91). Since the primary route of transmission of two other parvoviruses, parvovirus B19 and HBoV, is the respiratory tract, it is possible that parvovirus types 4 and 5 are also respiratory pathogens, although this has yet to be demonstrated. Mimivirus is a large DNA virus first discovered in *Acanthamoeba polyphagia*, and there is growing evidence that it may a true respiratory pathogen. La Scola et al. (154) recently reported that 9.7% of community-acquired pneumonia cases had antibodies to mimivirus, compared with 2.3% of healthy controls. Mimivirus DNA was found in bronchoalveolar lavage specimens from 1 of 32 intensive care unit patients in France, while 5 of 26 intensive care unit patients had serological evidence of mimivirus infection (none of 50 controls were antibody positive). In another study of 496 pneumonia patients tested for mimivirus using real-time PCR, this virus was not detected in any of the patients included in the study (55). Further studies will therefore be required to determine whether mimivirus and parvovirus types 4 and 5 are clinically important respiratory pathogens in humans.

Parvovirus type 4 and 5 DNAs have been detected by conventional or nested PCR targeting the NS-1 gene (125, 179). Mimivirus infection has been diagnosed serologically by microimmunofluorescence using antigen dots on microscope slides, PCR, and virus isolation in amoeba cultures (154). No commercial tests are available for parvovirus types 4 and 5 or mimivirus.

TABLE 12. Molecular tests for detection of bocavirus[a]

Assay format	Gene target(s)	Sensitivity (%)	Specificity (%)	Description	Reference
PCR	NP-1 (354 bp)	ND	ND	PCR detected 17 positive specimens out of 540 specimens tested; PCR primer may not be optional; no performance data given	4
PCR	NP-1	ND	ND	PCR detected 18 positive specimens out of 324 specimens tested	241
PCR	NP-1 (354 bp)	ND	100	PCR detected 22 positive specimens out of 425 specimens tested; all confirmed as true positive results by sequencing; 0 out of 96 specimens from asymptomatic children were positive	134
Seminested PCR	NP-1 (368 bp), VP-1 and VP-2 (980 bp)	ND	ND	Two seminested PCR assays detected 38 positive specimens out of 341 specimens evaluated	244
Multiplex PCR	NS-1	ND	ND	Multiplex PCR for 11 viruses was more sensitive than culture and DFA for 6 viruses; detected 58 positive specimens out of 515 specimens tested	42
Nested and nonnested PCR	NS-1, NP-1	ND	98.1	Nested PCR detected 54 positive specimens out of 924 specimens; 53/54 were confirmed positive with second primer set	178
Real-time PCR	NS-1 (88 bp), NP-1 (81 bp)	ND	ND	Real-time PCR targeting NS-1 and NP-1 genes; equivalent sensitivity to that of end-point assay; LLOD of 10 GE; 8-log dynamic range; 18/71 specimens could not be confirmed using second PCR	167
Real-time PCR	NP-1	ND	ND	Real-time LightCycler PCR assay more sensitive than end-point assay, detecting 33% more positive specimens; LLOD of 10 GE	3
PCR	NS-1	ND	ND	PCR detected 9 positive specimens out of 200 specimens collected over 7 yr	173
PCR	NP-1, NS-1	ND	ND	PCR detected 39 positive specimens out of 1,060 specimens tested; all positive results were confirmed by second primer set	176
PCR	NP-1 (354 bp)	ND	ND	PCR detected 21 positive specimens out of 261 specimens tested	195
PCR	NP-1 (291 bp)	ND	ND	PCR detected 15 positive specimens out of 315 specimens tested	8
Real-time PCR	NP-1 (354 bp)	100	ND	Real-time LightCycler assay detected 182 positive specimens out of 1,474 specimens evaluated; positive results were confirmed using second PCR targeting the NS-1 gene; LLOD of 17 copies	10
PCR	NP-1 (354 bp)	ND	ND	PCR detected 57 positive specimens out of 312 specimens tested; 14 amplicons were cloned and sequenced, revealing 5 variants	129

[a] ND, not determined; NS-1, nonstructural protein 1; NP-1, nucleoprotein 1; VP, capsid protein.

QUALITY ASSURANCE AND INTERPRETATION OF RESULTS

Many factors will impact the performance of NAATs for respiratory viruses. Preanalytical factors such as specimen collection, transport, and nucleic acid extraction will influence the ability of all amplification techniques to detect viral nucleic acid. Extracting a larger volume of the specimen can make a big difference in the performance of some assays, and the optimal sample volume to be extracted needs to be determined empirically for both the type of clinical specimen and the extraction protocol in use (210). Since most respiratory viruses are RNA viruses, which accumulate mutations more frequently than DNA viruses due to the lower fidelity of RNA-dependent RNA polymerase, the emergence of new variants (serotypes or genotypes) might be missed by a particular set of oligonucleotide primers or probes. For this reason, all NAATs, whether

in-house or commercial, should be monitored regularly to ensure that current strains are detected. Caution must be exercised when interpreting NAAT results for detecting respiratory viruses in clinical specimens using non-FDA-cleared assays. The use of laboratory-developed assays including those that use ASRs requires a complete evaluation prior to use in the clinical laboratory. The use of a second confirmatory NAAT is good laboratory practice when a new test is being introduced into the laboratory, especially for emerging pathogens (SARS-CoV or H5N1) with the potential for significant morbidity and mortality. A negative RT-PCR test on a nasopharyngeal specimen from a patient with influenza virus infection could result in nosocomial outbreaks, with significant costs to the health care system. A positive RT-PCR result obtained with a non-FDA-approved test should be confirmed either by performing a second PCR test, by testing a follow-up specimen, or by sending the specimen to a reference laboratory. Follow-up specimen testing and sending specimens to a reference laboratory for confirmation are important quality control procedures. The use of follow-up patient specimens may increase the sensitivity and overall accuracy of PCR for making a specific diagnosis. Positive and negative controls should be included in every run, and internal amplification controls (spiked RNA) are useful for detecting amplification inhibitors in specimens. Clinical laboratories should use the Laboratory Response Network as well as the CDC to confirm important positive findings for emerging viruses such as SARS and avian influenza H5N1 virus. What does a positive NAAT result mean clinically: is the patient currently infected, is the patient shedding viable virus, and is he or she infectious? NAATs will not help in assessing infectious virus, since these tests detect viral nucleic acid only. Longitudinal studies employing both culture and NAAT will be required to elucidate the period of shedding of infectious virus and nucleic acid, respectively, for both "old" and newly discovered viruses. The same questions apply for dual infections: is the patient infectious for one virus or both viruses? The clinical importance of dual infections has not been determined, and clinical studies will be required to determine whether patients with dual infections have a poorer prognosis.

CONTRIBUTION OF MOLECULAR TESTING

Molecular testing has greatly improved the laboratory's ability to diagnose viral respiratory tract infections. Without exception, NAATs have been shown to be more sensitive than non-nucleic-acid-based tests. The increased sensitivity means that infected patients will be diagnosed more accurately and more often, especially at times during the course of their infection when they are shedding low levels of virus that would be missed by nonmolecular tests. The benefit of a more accurate diagnosis is fourfold: first, it benefits the patient in terms of receiving the appropriate antiviral drugs such as oseltamivir in the case of influenza virus; second, it assists infection control practitioners in providing appropriate infection control measures such as droplet containment when necessary to minimize the risk of nosocomial spread; third, it can stop the search for a diagnosis even if there is no beneficial antiviral agent for the respiratory virus that was found; and fourth, it provides more accurate information to public health authorities regarding what viruses are circulating in the community so that they can adjust public health policy accordingly. For potentially catastrophic global events such as the emergence of SARS and avian influenza H5N1 virus, accurate diagnostic tests such as NAATs have played a crucial role in identifying the agent and tracking the outbreaks and will likely play a key role in the case of future pandemics.

The development and adoption of multiplex PCR tests for respiratory viruses will have an even greater benefit over the next few years since multiplex tests offer laboratories the ability to detect a wide range of viral infections, which was not previously possible. These tests will allow laboratories to report infections with conventional viruses such as rhinovirus as well as newly discovered viruses such as HCoV-NL63 and -HKU1 and HBoV using a single test. Testing for multiple viruses in a single test may provide a savings of resources including technologist time and expendables and cost less than the aggregate cost of performing multiple uniplex PCR tests. The implementation of robotics in large-volume laboratories should decrease costs further since a significant portion of the cost is technologist time. Dual respiratory virus infections are poorly detected using DFA and SVC methods because of subjective IF readouts. Dual and even triple infections are routinely reported using multiplex PCR and have accounted for between 8 and 11% of positive specimens (45, 177, 211). The ability to easily detect dual infections provides the means and impetus for studies to examine the clinical importance of dual infections and in particular whether certain individuals are at greater risk for dual infections or whether they result in a poorer outcome for the patient. Finally, multiplex tests will contribute to our understanding of the epidemiology of viral respiratory tract infections, as many specimens can be tested for multiple viruses, providing a wealth of new information on seasonality, geographical distribution, and risk groups (177).

As diagnostic testing continues to evolve, we can expect to see more multiplex PCR tests developed for use in the clinical laboratory. These will include low- and medium-density microarrays, including both fluidic microarrays and DNA chips. The development of multiplex PCR assays for detection of multiple respiratory viruses (176, 317) sets the stage for future multiplex assays for the detection of not only respiratory viruses but enteric viruses and other groups of viruses present in a clinical specimen. The xTAG RVP test (Luminex Molecular Diagnostics, Toronto, Ontario, Canada) has been approved by the FDA for the detection of 12 respiratory viruses, while the ProFlu+ assay has been approved for the detection of influenza A virus, influenza B virus, and RSV. These tests, plus the ResPlex II (Qiagen), MultiCode-PLx (EraGen Biosciences), and the Nanogen RVA assays, which have not been FDA cleared, offer clinical laboratories exciting new tools for the detection of respiratory viruses. The use of multiplex NAATS or the use of multiplex single-target assays has increased the diagnostic yield for respiratory viruses by 30 to 50% over that by conventional test methods (18, 176). FDA clearance will, however, be required before these other tests can be used as in vitro diagnostic devices. Comparative clinical performance data for some of these tests are emerging (18, 221) and are eagerly awaited so that clinical laboratories will know the true sensitivities and specificities of the various assays and how they compare. As these new tests are evaluated and their performances are critically monitored and compared, clinical labo-

ratories will have effective new approaches for detecting respiratory viruses and be able to provide clinicians with new and important information to assist with patient management decisions.

For most if not all respiratory viruses, molecular detection of viral nucleic acid is the most sensitive diagnostic approach. It is easy to envisage that the current "gold standard" for detecting conventional repiratory viruses, viz., culture and DFA, will be challenged and eventually replaced by NAATs. Few people would argue that the gold standard for emerging viruses (SARS CoV and avian influenza virus H5N1) is already nucleic acid amplification. The recent discovery over the past 6 years of six new respiratory viruses that infect humans has presented enormous challenges for virology laboratories to develop, evaluate, and implement sensitive and specific tests for the detection of these emerging viruses. It is likely that new respiratory viruses will continue to be discovered in the years ahead and that new diagnostic tests will be required to both determine the impact of these agents and assist clinicians in the management of their patients. The costs associated with running NAATs for the detection of respiratory viruses may be an extra burden on the laboratory, and careful cost-benefit studies will need to be conducted to determine whether the increased costs of molecular tests is offset by benefits to the hospital, viz., a reduction in unnecessary patient workups and knowing in a timely fashion what respiratory virus is being dealt with. These studies are presently ongoing and the results are eagerly awaited. Given the scarcity of FDA-approved molecular tests for respiratory viruses, clinical laboratories will need to apply careful criteria for the evaluation of available ASRs and RUO tests to be assured that they are performing as expected. The good news for laboratories is that several companies are developing multiplex NAATs together with new platforms for the detection of multiple respiratory viruses.

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