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REVIEW

Recent advances in the detection of respiratory virus infection in humans

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Abstract

Respiratory tract viral infection caused by viruses or bacteria is one of the most common diseases in human worldwide, while those caused by emerging viruses, such as the novel coronavirus, 2019-nCoV that caused the pneumonia outbreak in Wuhan, China most recently, have posed great threats to global public health. Identification of the causative viral pathogens of respiratory tract viral infections is important to select an appropriate treatment, save people's lives, stop the epidemics, and avoid unnecessary use of antibiotics. Conventional diagnostic tests, such as the assays for rapid detection of antiviral antibodies or viral antigens, are widely used in many clinical laboratories. With the development of modern technologies, new diagnostic strategies, including multiplex nucleic acid amplification and microarray-based assays, are emerging. This review summarizes currently available and novel emerging diagnostic methods for the detection of common respiratory viruses, such as influenza virus, human respiratory syncytial virus, coronavirus, human adenovirus, and human rhinovirus. Multiplex assays for simultaneous detection of multiple respiratory viruses are also described. It is anticipated that such data will assist researchers and clinicians to develop appropriate diagnostic strategies for timely and effective detection of respiratory virus infections.

KEYWORDS
adenovirus, coronavirus, diagnostic methods, influenza virus, respiratory syncytial virus, respiratory viral infection, rhinovirus

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1 | INTRODUCTION

Acute respiratory disease (ARD) accounts for a large proportion of all acute morbidities, as well as mortalities, worldwide, among which acute viral respiratory tract infection is the leading cause (appropriate 80%).[1] The major viral pathogens include influenza virus, respiratory syncytial virus (RSV), coronavirus, adenovirus, and rhinovirus. In children below the age of five, the combined global mortality of only influenza and RSV reaches 300 000 deaths each year.[2] Other respiratory viruses, such as adenovirus and rhinovirus, are associated with lower mortality, but significant morbidity, causing a huge economic burden.[3] The highly pathogenic emerging and reemerging coronaviruses that may cause epidemics or pandemics, such as severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV), have posed great threat to global public health. Most recently, a novel coronavirus 2019-nCoV (https://www.who.int) has caused the pneumonia in 41 confirmed cases of patients, including 7 in serious condition, 1 dead, and 2 recovered (https://www.shine.cn/news/nation/2001119576/). The full genomic sequence of this coronavirus was released on January 10, 2020 (http://virological.org/t/initial-genome-release-of-novel-coronavirus/319?from=groupmessage&isappinstalled=0), which is more than 82% identical to those of SARS-CoV and bat SARS-like coronavirus (SL-CoV).[4]

Accurate and fast diagnosis of the causative viral pathogens is important to select the appropriate treatment, save people's lives, stop the epidemics, and reduce unnecessary use of antibiotics. All viruses mentioned above can cause both upper and lower respiratory tract infections, as well as overlapping clinical presentations, making it difficult for physicians to distinguish the causative agents without a solid laboratory analysis. Conventional diagnostic methods, such as viral culture and direct/indirect immunofluorescence assay (IFA), are time-consuming and labor intensive with limited sensitivity. Rapid and accurate diagnosis of respiratory viruses can help in epidemiologic monitoring, along with taking effective prevention steps and implementing appropriate antiviral therapies. Over the past decades, an evolution in viral diagnostic testing has been seen, all the way from conventional approaches to rapid antigen detection. More recently, highly sensitive nucleic acid amplification tests (NAAT) and point-of-care tests (POCT) have been developed.[2]

In this review, we describe various approaches currently available, or under development, for diagnosis of common respiratory virus infections in humans. It is anticipated that these data will assist clinical laboratories to rapidly and accurately diagnose respiratory viruses, thus providing physicians with essential information for timely and appropriate treatment of patients. The commonly used diagnostic approaches for influenza virus, RSV, coronavirus, adenovirus, and rhinovirus are summarized in Table 1 and described in detail in the following review.

2 | INFLUENZA VIRUS AND ITS DIAGNOSTIC APPROACHES

Influenza viruses belong to the family of *Orthomyxoviridae*. This family consists of five genera (influenza A virus, influenza B virus, influenza

TABLE 1 Respiratory viruses and their specific diagnostic approaches.

Virus type	Family and Genus	Diagnostic approaches	References
Influenza virus	*Orthomyxoviridae family & Influenza virus A, Influenza virus B, Influenza virus C, Influenza virus D, Isavirus, Quaranjavirus and Thogotovirus genus*	Viral culture; IFA; ELISA-based test; PCR-based (reverse transcriptase-PCR, LAMP); DNA-microarray-based; sequencing-based tests	5–14
Human RSV	*Paramyxoviridae family & Pneumovirus genus*	IFA; ELISA-based test; DFA; LFIA (Remel Xpect, Binax Now RSV, BD Directigen EZ RSV, QuickLab RSV, Respi-Strip); real-time PCR based (TaqMan PCR, Locked nucleic acid (LNA)-based one-tube nested real-time (OTNRT) RT-PCR); RT-RAA assay; RT-SIBA	15–27
Coronavirus	*Coronaviridae family & Alphacoronavirus, Betacoronavirus, Deltacoronavirus and Gammacoronavirus genus*	RT-PCR; rRT-PCR (PowerCheck, DiaPlexQ, Anyplex, AccuPower, LightMix, UltraFast); RT-LAMP; Real-time RT-LAMP	28–34
Adenovirus	*Adenoviridae family & Atadenovirus, Aviadenovirus, Mastadenovirus and Siadenovirus genus*	Viral culture; Indirect ELISA; IFA; LAT; EIA; Real-time PCR based (RealStar® Adenovirus PCR kit 1.0, in-house hAdV qPCR assay)	35–39
Rhinovirus	*Picornaviridae family & Enterovirus genus*	CFT; HI; IFA; ELISA; Semi-nested RT-PCR assay; One-step Panenterhino/Ge/08 real-time RT-RCR assay; WGS-based assays	40–43

Abbreviations: CFT, complement fixation test; DFA, direct fluorescent assay; EIA, enzyme-immunoassay; ELISA, enzyme-linked immunosorbent assay; HI, hemagglutination inhibition; IFA, immunofluorescence assay; LAMP, loop-mediated isothermal amplification; LAT, lateral-flow test; LFIA, lateral-flow immunochromatographic assay; LNA, locked nucleic acid; OTNRT, one-tube nested real-time; PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction; rRT-PCR, real-time reverse transcription-polymerase chain reaction; RT-LAMP, reverse transcription loop-mediated isothermal amplification; RT-RAA, reverse transcription recombinase-aided amplification assay; RT-SIBA, reverse transcription strand invasion-based amplification; WGS, whole genome sequencing.

C virus, Thogotovirus, and Isavirus), which are classified based on internal nucleoprotein (NP) and matrix (M) proteins. Among these genera, only influenza A and influenza B viruses cause clinical diseases. Influenza B viral infections usually cause localized outbreaks, whereas influenza A virus is the primary pathogen for human infections and, thus, the major cause of large influenza epidemics and pandemics.[44] Based on the glycoproteins hemagglutinin (HA) and neuraminidase (NA), which are located on the viral surface, influenza A viruses are divided into various subtypes. Until now, 18 HA (H1-H18) and 11 NA (N1-N11) subtypes have been identified.[44] A number of diagnostic approaches, including virus isolation, as well as some emerging molecular-based approaches, have been used to detect influenza viruses in clinical laboratories.

2.1 | Traditional approaches for influenza virus detection

Viral culture is the gold standard for diagnosing influenza viral infections. This approach includes inoculation of the corresponding cell lines, such as Madin Darby canine kidney (MDCK), A549, and rhesus monkey kidney (LLC MK2), with clinical samples, propagation for seven to 10 days to monitor the development of cytopathic effect (CPE), and final confirmation of influenza virus infection by hemadsorption using erythrocytes, specific antibody staining or immunofluorescence microscopy.[5] IFA has been used since the 1960s. It directly stains respiratory epithelial cells derived from nasopharyngeal aspirates or swabs with fluorescently labeled influenza virus-specific antibodies.[5] Currently, several FDA-approved enzyme-linked immunosorbent (ELISA)-based tests are available for diagnosis of influenza virus infections. However, ELISA-based tests often show lower sensitivity compared with nucleic acid-based methods. A novel immunoassay based on europium nanoparticles has been developed to rapidly detect 29 strains of influenza A and 10 strains of influenza B viruses with high sensitivity and specificity.[6]

2.2 | Molecular–based approaches for influenza virus detection

The above-described conventional diagnostic methods generally have lower sensitivity and specificity relative to molecular methods. With the development of laboratory approaches for virus diagnosis, isolation of viral pathogens from secretion samples has become much easier than before.

The rapid influenza diagnostic test (RIDT) offers a faster approach for detecting both influenza A and B viruses. However, it has limited ability to further identify the subtypes of influenza viruses. In addition, its test performance (ie, sensitivity and specificity) varies widely. For example, in November 2009, 290 suspected influenza patients were analyzed using direct antigen EZ Flu A+B kit. The sensitivity and specificity of RIDT were 40.5% and 94.5%, respectively.[45] NAAT assays based on polymerase chain reaction (PCR) detect virus-specific genetic materials, rather than viral antigens or antibodies. Therefore, optimal extraction of viral genetic materials is required. One of the advantages of NAAT over RIDT is that NAAT is able to identify different subtypes of influenza viruses. Trombetta's group found that NAAT shows much higher sensitivity for both influenza A and influenza B viruses, despite its relative lower specificity.[7] A variety of NAAT assays, such as reverse transcriptase-PCR, loop-mediated isothermal amplification-based assay (LAMP), DNA-microarray-based and sequencing-based tests, are appearing for diagnosis of influenza viral infections in humans.[5] Using the LAMP approach, Poon's group demonstrated 100% sensitivity for detection of seasonal influenza A H1N1 and H3N2 viruses from clinical specimens with analytical sensitivity of 10 copies per reaction.[8] Compared with the RT-PCR-based assay, a real-time reverse transcription LAMP-based assay (RT-LAMP) showed a sensitivity of 97.8% and a specificity of 100% for detecting 2009 H1N1 pandemic virus.[9] LAMP-based assays have also been used to detect the highly pathogenic H5N1 and H7N9 avian influenza viruses, having comparable, or higher, sensitivities than RT-PCR-based approaches.[10–12] However, the high cost of NAAT, the complexity of the equipment required, and the need for trained professionals make NAAT assays less practical in areas with limited resources.

In addition to the above approaches, the DNA-microarray-based approach has been developed to detect influenza viruses, offering a novel tool for accurate and rapid diagnosis of influenza epidemics or pandemics. This assay uses multiple oligonucleotides specially designed to target conserved sequences encoding HA, NA and M proteins of influenza A and B viruses.[5] For instance, Zhao's research group has developed a gold nanoparticle-based genomic microarray assay, which is able to differentiate H5N1 viruses from the major seasonal influenza A viruses (H1N1, H3N2). This can be explained by oligonucleotides which are designed on the basis of consensus sequence of the HA and NA genes of the H5N1 virus and M gene of H1N1, H3N2, and H5N1 viruses.[13] The same group has expanded the platform and designed specific oligonucleotides for HA, NA and M genes of the 2009 pandemic H1N1 virus, enabling the detection of the 2009 pandemic H1N1 virus and distinguish it from other influenza A viruses.[46]

Particularly, a combinational diagnostic platform is reported using nanomicroarray for screening and multiplex next-generation sequencing (NGS) assays for confirmation to simultaneously identify and characterize influenza A and B viruses in a single sample.[47] Recently, a high-throughput whole genome sequencing (WGS) method with the MinION portable sequencer was developed to test influenza A and B viruses, as subsequently validated by the Illumina MiSeq platform. The overall accuracy, precision, as well as recall rates, were 99.95%, 97.88%, and 89.41%, respectively, from 1D reads and 99.97%, 99.86%, and 93.28%, respectively, from $1D^2$ reads.[14]

3 | HUMAN RESPIRATORY SYNCYTIAL VIRUS AND ITS DIAGNOSTIC APPROACHES

Human RSV is a nonsegmented, negative-sense and single-stranded RNA virus of the *Paramyxoviridae* family. The genome of RSV includes ten genes that encode eleven proteins. RSV can be classified into

subgroups A and B according to the genome sequence and the reactivity of monoclonal antibodies (mAbs) to the surface glycoprotein (G) and fusion protein (F).[48,49] RSV is a leading cause of severe respiratory disease in immunocompromised populations, such as infants and elderly populations, with significant morbidity and mortality worldwide. Early and accurate RSV diagnosis is crucially important for appropriate treatment.

3.1 | Traditional approaches for human respiratory syncytial virus detection

ELISA and immunofluorescence assays are traditional assays to identify RSV. However, a modified ELISA method has been developed, targeting RSV F protein and it can detect RSV within 25 minutes at low cost.[15] The immunofluorescence assay can rapidly detect RSV antigens using a fluorescence-tagged primary or secondary antibody. For example, the direct fluorescent antibody assay (DFA), which requires a certain number of cells in the specimen, with a sensitivity and specificity of 94% and 96.8%, respectively, is widely used for detection of RSV in clinical laboratories because of its simplicity and rapidity. For this reason, this assay has particular use in resource-limited countries since it can potentially eliminate prolonged hospitalization and unnecessary use of antibiotics.[16]

Semiconductor quantum dots can be used for biological and biomedical applications because of their unique size-dependent optical and electronic features. The assay detects RSV F protein using thioglycolate (TGA)-coated cadmium telluride (CdTe) particles, which are bioconjugated with RSV anti-F protein mAb.[17] It overcomes some of DFA's disadvantage, such as relatively low sensitivity, because of the background staining, and the rapid fading of the dye. Also, this assay is more sensitive than RT-PCT. By probing F and G proteins with QDs, confocal microscopy could detect the progression of RSV infection in the HEp-2 cell line, and this method was found to be more sensitive compared to RT-PCR.[18]

Lateral flow immunoassay (LFIA) is another rapid RSV detection method based on an immunochromatographic technique using the samples of nasal washes or aspirates. Many LFIA kits are now available in the market, such as BD Directigen EZ RSV, Binax Now RSV, RSV Respi-Strip, Remel Xpect, and QuickLab RSV Test.[19–21] The sensitivity and specificity of the abovementioned kits are normally higher than 90% and 95%, but they differ by manufacturer.

3.2 | PCR-based approaches for human respiratory syncytial virus detection

The PCR method is based on the nested RT-PCR technique involving the outer and inner primers designed from the F gene of RSV-A or -B. This method has been developed in both circle time and operability in adult infections.[22] Therefore, it can be used to detect samples with low viral titers and sensitively perform identification using antigen-based detection approaches.

The following novel PCR detection methods have been established by modification of the conventional PCR approach. For example, real-time quantitative PCR (RT-qPCR) is a rapid, specific, and sensitive TaqMan PCR method for detection, subgrouping, and quantitation of pathogens. This assay increases the sensitivity of conventional PCR. It needs two sets of primer-probe pairs, which come from the nucleotide sequence of nucleocapsid (N) gene or Fusion (F) gene targeting RSV-A and RSV-B, respectively.[23,24] A quantitative TaqMan PCR assay was once used to detect 175 nasopharyngeal aspirates obtained from children with respiratory symptoms in Hong Kong, and it detected 36 RSV-positive samples, including 10 as RSV-A and 26 as RSV-B. In contrast, a cell culture-based assay only identified 21, and an immunofluorescence assay identified 32 RSV-positive specimens, all of which were among those identified by the TaqMan PCR assay, suggesting the greater accuracy and sensitivity of the TaqMan PCR assay.[24] In addition, locked nucleic acid (LNA)-based one-tube nested real-time (OTNRT)-PCR is an assay with very high sensitivity and low incidence of cross-contamination for detection of RSV.[25] A total of 143 nasopharyngeal aspirate samples that tested RSV-negative by qRT-PCR were confirmed as RSV-positive by sequencing the OTNRT-PCR products,[25] indicating that OTNRT-PCR is more sensitive than RT-qPCR for detection of RSV in clinical samples. Moreover, the rapid reverse-transcription recombinase-aided amplification (RT-RAA) assay was developed as a molecular-based diagnostic method to detect subgroup RSV A and B genomes in clinical specimens. This method mainly utilizes an enzyme mixture, including single-strand DNA binding protein (SSB), recombinase UvsX, and DNA polymerase, to detect RNA amplicons of RSV.[26] It is performed at 39°C in less than 30 minutes with high specificity. Furthermore, reverse transcription strand invasion-based amplification (RT-SIBA) is reverse transcription isothermal nucleic acid amplification for rapid detection of RSV with good sensitivity since it can detect as few as 10 copies of RSV RNA within 20 minutes.[27] RT-SIBA does not need highly purified RNA for detection of RSV, which can reduce the complexity of specimen preparation and shorten the total detection cycle in clinical specimens.

4 | CORONAVIRUS AND ITS DIAGNOSTIC APPROACHES

Coronaviruses belong to the family of *Coronaviridae*. Bats have been recognized as natural reservoir and vectors of a variety of coronaviruses and the viruses have crossed species barriers to infect humans and many other different kinds of animals, including avians, rodents, and chiropters.[50,51] Coronavirus may cause respiratory and neurological diseases.[52] So far, six human coronaviruses (HCoVs) have been identified, including HCoV-229E, HCoV-HKU1, HCoV-OC43, HCoV-NL63, severe acute respiratory syndrome coronavirus (SARS-CoV), and Middle East respiratory syndrome coronavirus (MERS-CoV).[53] MERS-CoV is a human coronavirus first reported in June 2012 that can cause human respiratory diseases.[52] It has been classified as a lineage C β-coronavirus, and its structure comprises a ~30 kb positive-sense, single-stranded RNA genome, which is closely

related to the lineage C β-coronaviruses of *Tylonycteris* bat CoV HKU4 and *Pipistrellus* bat CoV HKU5.[54] By genomic analysis of lineage C β-coronaviruses, human MERS-CoV has shown high similarities to MERS-CoV derived from camels with >99.5% nucleotide identities, suggesting that the human and camel isolates belong to the same coronavirus species.[4] As of September 30, 2019, 2468 laboratory-confirmed cases of MERS-CoV infections, including 851 deaths in 27 countries, have been reported to WHO (https://www.who.int/emergencies/mers-cov/en/). Because no commercial vaccines or therapeutic treatments are currently available for MERS, rapid and accurate diagnosis of MERS-CoV is important for the prevention of its transmission and outbreaks.

Current diagnostic tests for coronavirus include RT-PCR, real-time reverse transcription PCR (rRT-PCR), reverse transcription loop-mediated isothermal amplification (RT-LAMP), as well as real-time RT-LAMP.[51,55–58]

A duplex RT-PCR method has been developed based on primers and probes targeting the conserved spike S2 gene of SARS-CoV and MERS-CoV. By using pUC57SARS-pS2 as a template for SARS-CoV and pGEM-MERSS2 as a template for MERS-CoV, respectively, adequate detection limits of 50 to 100 copies/mL were achieved.[28] The singleplex RT-iiPCR assays are designed to detect MERS-CoV envelope gene (upE) and open reading frame 1a (ORF1a) genes separately. Compared to the reference singleplex RT-qPCR assay, the sensitivities of the singleplex MERS-CoV ORF1a and upE RT-iiPCR assays are 99.03% and 100%, respectively.[29]

Six commercially available MERS-CoV RNA detection kits based on rRT-PCR are available, including AccuPower (Bioneer, Korea), Anyplex (Seegene, Korea), DiaPlexQ (SolGent, Korea), LightMix (Roche Molecular Diagnostics, Switzerland), UltraFast kits (Nanobiosys, Korea) and PowerChek (Kogene Biotech, Korea). The PowerChek MERS Real-time PCR, Anyplex II MERS-CoV (upE) Real-time Detection and DiaPlexQ MERS Virus Detection kits consist of two steps. A single gene targets the upstream region of the upE region for screening, and multiple genes target both upE and ORF1a regions for final confirmation.[30] The AccuPower MERS-CoV Real Time RT-PCR, LightMix Molecular Dx MERS-CoV upE/ORF1a, and UltraFast LabChip MERSCoV Real-time PCR kits use two single gene-targeting reagents to detect the upE and ORF1a genes simultaneously.[30] No cross-reactivity has been reported with other respiratory viruses. Based on the validation tests, including 28 specificity panels and 9 clinical specimens, the sensitivity and specificity of all these rRT-PCR kits for detecting upE and ORF1a reach 100% (95% confidence interval [CI], 0.60-1.00) and 100% (95% CI, 0.79-1.00), respectively. Based on results from the high inhibition panel, AccuPower and PowerChek have lowest sensitivity to the presence of PCR inhibition.[29] Therefore, the overall sensitivity and specificity of the above six rRT-PCR kits are sufficient for diagnosing MERS-CoV infection.

In the RT-LAMP assay, two primer sets were constructed with one set targeting the N gene and one set targeting the ORF1a gene. Both sets have shown high efficiency in amplifying target sequences derived from different MERS-CoV strains with no cross-reactivity observed with other respiratory viruses.[31] Huang's research group has established a nucleic acid visualization technique that combines the RT-LAMP technique and a vertical flow visualization strip (RT-LAMP-VF) to detect the N gene of MERS-CoV.[52] The RT-LAMP-VF assay can detect 2×10^1 copies/μL of synthesized RNA transcript and 1×10^1 copies/μL of MERS-CoV RNA without cross-reactivity to multiple SARS-related-CoVs, including HKU1, HKU4, OC43 and 229E, thus exhibiting high specificity.

RT-LAMP and RT-LAMP-VF mentioned above are assays for rapid and accurate detection of MERS-CoV infection. Bonhan Kooa's team designed an arch-shaped multiple-target sensor capable of rapid pathogen identification using direct amplification in clinical samples. The method can detect a variety of viruses including MERS-CoV with high sensitivity and specificity and is able to distinguish MERS-CoV from HCoV in clinical samples within 20 minutes.[32] The TaqMan probe-based one-step rRT-PCR assays provide rapid and sensitive internal diagnostic detection of MERS-CoV by detecting the upE and open reading frame (ORF) 1b. This method is reliable, specific and reproducible. In addition, different from the traditional two-step sample detection method, the one-step method combines two steps into one step, thus increasing the sensitivity of the measurement, for example, <10 and ≦50 copies of RNA template per reaction for upE and ORF1b, respectively.[33] The MAb-based rapid NP detection method for MERS-CoV was used for preliminary rapid screening of MERS-CoV infection due to its high specificity but moderate sensitivity. The detection limit of this assay is about $10^{3.7}$ to $10^{4.2}$ $TCID_{50}$/mL of MERS-CoV, and the sensitivity is about 25- to 100-fold lower than that of antigen capture ELISA.[34]

5 | HUMAN ADENOVIRUS AND ITS DIAGNOSTIC APPROACHES

Adenovirus (AdV) infections are now regarded as a significant source of human or animal morbidity and mortality. Human adenovirus (hAdV) infections are readily transmittable, infecting all age groups, especially infants and the elderly, as well as those with immunodeficiency and organ transplantation.[59] HAdV has a non-enveloped spherical structure with a diameter of 70 to 100 nm. The external shell consists of 252 capsomeres, including 240 hexons and 12 pentons, which are composed of an icosahedral viral capsid. The capsomeres are arranged on 20 triangular surfaces, making the shell form 30 edges and 12 vertices. The genome of hAdV exists in the capsid in the form of linear double-stranded DNA with sizes ranging from 34 to more than 37 kb. The genome contains the early expressed E1~E4 genes associated with replication of hAdV, intermediate gene of IX and IVa2, and late region genes L1~L5 associated with the production of mature virions.[60] HAdV belongs to the *Adenoviridae* family, and it has been classified into seven species (A to G) with over 50 serotypes based on hexon and fiber protein characteristics, relative nucleic homology, immunochemical responses, biological properties, and phylogenetic relationships.[35,61,62] This subdivision has some clinical relevance, essentially because different hAdVs differ in tissue orientation and disease type. HAdV can cause multiple diseases, such as respiratory

disease (hAdV-B, -C, and -E), gastroenteritis (hAdV-F and -G), keratoconjunctivitis (hAdV-B and -D), and meningoencephalitis (hAdV-A, -B, and -D).[59,63] Therefore, effective and rapid diagnosis of hAdV ahead of its progression will provide for effective monitoring of hAdV infection and guarantee timely and effective treatment of hAdV-associated diseases.

5.1 | Tratitional approaches for human adenovirus detection

The isolation of adenovirus after growth in cell culture and its immunological detection are generally thought to be the "gold standard."[36] Samples, such as throat swabs and stool specimen, collected from patients are treated overnight with antibiotics, centrifuged, and supernatant inoculated with susceptible cells (Hep-2, Graham 293, or A549). CPE is observed after cell incubation at 37°C, and then the hAdV is isolated. The serotype of hAdV can be determined by detecting the antigenic determinant of penton proteins by the hemagglutination inhibition (HI) test. HAdV can also be typed by detecting the antigenic determinant of hexon proteins by neutralizing test (NT). However, these methods are time-consuming, require experienced technicians to analyze the results, and the results are still uncertain. Different types of hAdV infection can cause different diseases and different symptoms; thus, detection of antigens and antibodies can make the diagnosis of hAdV more rapid and sensitive. Indirect ELISA can detect the IgA, IgM and IgG antibodies of hAdV in human serum or plasma. Immunofluores (IF), latex agglutination test (LAT) and enzyme immunoassay (EIA) are also common diagnostic methods accepted by most clinical laboratories because of their simple and rapid characteristics.

5.2 | Molecular-based approaches for human adenovirus detection

Emerging molecular-based detection methods have gradually replaced traditional detection technology in many laboratories and clinical practices, providing reliable new methods for the diagnosis of hAdV.[60]

Specific antigenic determinants on the hexon surface of hAdV can be identified by neutralizing antibodies. The antigenic determinants of hexon proteins have two loops, L1 and L2, which are divided into seven hypervariable regions (HVR), among which HVR 1 to 7 has become the newest method of choice for adenovirus typing. Madisch developed a two-step diagnostic method that can classify 51 serotypes by amplification and sequencing of the amplification elements on L1 and L2.[37] Shimada studied a more conservative hexon gene on HVR, which can type 44 serotypes, but it is limited to the hAdV serotypes that can lead to corneal conjunctivitis.[38] Real-time PCR is a sensitive and quantitative technique to diagnose hAdV infections. A real-time PCR with consensus primer and probe for a conserved region of the hexon gene has been designed to detect 51 prototypes. The sensitivity of the assay is ≤15 copies per run, and the linear range of quantitation was 1.5×10^1 to 1.5×10^8 copies/run.[39] The RealStar Adenovirus PCR Kit 1.0 is a real-time PCR-based diagnostic test for in vitro detection and quantification of hAdV-specific DNA. Its sensitivity is 1.09 copies/μL (95%, 0.62-3.08 copies/μL), and it has no cross-reactivity against 35 human viral pathogens. Compared with the RealStar Adenovirus PCR Kit, the in-house qPCR assay is more sensitive and reliable for the detection and quantification of hAdV-specific DNA. The performance characteristics of this in-house qPCR have been evaluated against the RealStar Adenovirus PCR Kit, using 122 clinical specimens and 18 proficiency samples.[35] In this study, the in-house hAdV qPCR assay detected six hAdV species A to F, except species G. Using the RealStar Adenovirus PCR Kit as the reference, the sensitivity and specificity are 98.1% and 100%, respectively, for the in-house qPCR assay, and the correlation of the two assays is quite good ($R^2 = 0.984$). Furthermore, the in-house qPCR assay has a linear range up to 9 log10 copies/m, and the %CV values suggested quite low intra- /inter-assay variations.

6 | RHINOVIRUS AND ITS DIAGNOSTIC APPROACHES

Rhinoviruses (RVs) are RNA viruses belonging to the family *Picornaviridae* genus *Enterovirus*, which has been classified into 3 species (A to C) with over 160 serotypes.[64–67] RVs are small single-stranded RNA viruses with an icosahedral symmetrical capsid.[68] RVs are the most frequent cause of respiratory tract infections in both children and adults. RV-A and RV-C are common RV species causing lower airway illness, wheezing and acute asthma, and they can lead to more severe symptoms than RV-B in children.[67,69]

Culture isolation of virus combined with acid stabilization test is a classic method for RV diagnosis. However, this assay is time-consuming and laborious, limiting its value for clinical treatment. Human RV antibodies can be detected by immunological methods, such as the complement fixation test (CFT), HI test, IF, and ELISA. However, owing to the lack of suitable cross-reactive antigens to cover a large number of RV serotypes, the application of these methods is greatly limited. In contrast, the one-step, real-time PCR assay detects RVs by using a sequence detection system. This assay improves workflow, reduces preparation time, and eliminates cross-contamination that might be introduced during cDNA transfer from the reverse transcription reaction into the PCR reaction step.[40] Semi-nested RT-PCR assay is based on an extremely sensitive PCR to detect airborne RVs, which has a limit of detection (LOD) of approximately 0.8 median tissue culture infectious dose ($TCID_{50}$).[41] The one-step Panenterhino/Ge/08 real-time RT-RCR assay has been developed and validated by using an internal extraction control and serial dilutions of an in vitro-transcribed rhinovirus RNA reference standard.[42] The LOD and limit of quantification (LOQ) of this assay are 3.10 log copies/mL and 4.10 log copies/mL, respectively. The linearity is conserved from 4.10 to 9.10 log copies/mL, and intra-assay reproducibility is $r^2 = 0.999$.[42] With the rapid development of

WGS technology, more and more laboratories may perform WGS on human RV isolates clinically. With the discovery of hRV-C, the data volume of whole-genome hRV sequencing has been increased and improved significantly. This will help researchers and clinicians to understand the evolutionary and recombination of RV,[43] which may improve diagnosis.

7 | MULTIPLEX RESPIRATORY VIRUS DETECTION

Although single respiratory viruses can be detected in patients with symptoms, other respiratory viruses may also exist simultaneously. Children, especially those under 5 years of age, present with a higher frequency of coinfections.[70–72] Multiplex assays that contain more than one viral gene target in a single tube have the advantage of rapid detection of several potential viral pathogens simultaneously. Multiplex real-time PCR has allowed simultaneous detection of multiple respiratory viruses in a short time. Compared with the singleplex approach, the multiplex diagnostic approach has higher sample throughput (96 samples per run), shorter turnaround time (5 hours), and a smaller amount of sample requirement.[73]

The Luminex NxTAG Respiratory Pathogen Panel (NxTAG-RPP) is a new high-throughput multiplex real-time PCR system, which was upgraded from the xTAG Respiratory Viral Panel Fastv2 (RVPv2) assay.[73] This assay demonstrates good diagnostic performance on detection of multiplex respiratory pathogens. A total of 284 clinical respiratory specimens, including hAdV and RV, and 3 influenza A/H7N9 viral culture samples have been tested, and the overall diagnostic sensitivity of the NxTAG-RPP was 98.9% (95% CI, 97.2 to 99.7), while the specificity was 99.0% (95% CI, 98.6 to 99.2). This assay demonstrated 100% sensitivity for detection of hAdV.[73] A number of such assays have been developed, as described, and are summarized in Table 2.

Recently, Feng's group designed locked nucleic acid LNA-modified primers and developed a multiplex one-tube nested real-time RT-PCR (mOTNRT-PCR) assay that could detect RSV, hRV and human metapneumovirus (hMPV) simultaneously with higher sensitivity and lower cost compared to the individual RT-qPCR.[74] This assay was evaluated using 398 clinical samples, and the sensitivity for RSV, hRV, and hMPV was 5 copies per reaction. No cross-reactivity was observed among other common respiratory viruses, and the total detection time of this assay was 2.7 hours for 96 samples.

The Panther Fusion respiratory assay has been developed to detect multiple respiratory viruses, and it consists of three multiplex real-time PCR panels: Flu A/B/RSV, Paraflu (Parainfluenza), and hAdV/hMPV/RV.[75] The performance characteristics of this assay for the detection of Flu A/B, RSV, Paraflu 1 to 3, hMPV, RV, and hAdV have been evaluated using 395 nasopharyngeal (NP) and 104 lower respiratory tract (LRT) samples.[75] The Wilson-126 Score method was used to calculate the 95% CI for positive percent agreement (PPA) and negative percent agreement (NPA). For the NP samples, the Panther Fusion Assay has 100% PPA and 98.4% to 100% NPA for all targets. For the LRT samples, it had 100% PPA for all targets and 100% NPA for all targets, except hMPV (96.1%).[75]

Fast Track Diagnostics Respiratory pathogens 21 (FTD21 kit) is a commercial multiplex nucleic acid amplification assay. The FTD21 kit has been evaluated using 665 samples from patients with influenza-like illness. This kit has sensitivity of 90.7% and specificity of 100% compared to the RealStar 1.0 kit. In addition, upon modification of the FTD21 kit, the sensitivity increased to 97.3%.[76]

A GeXP-based multiplex RT-PCR assay (GeXP assay) has been developed to detect 16 different respiratory viruses simultaneously.[77] Seventeen sets of chimeric primers were designed to initiate the RT-PCR, and another pair of universal primers was used for subsequent cycles of RT-PCR.[77] The GeXP assay achieves a sensitivity of 20 to 200 copies for a single virus, and when all 16 premixed viral targets were present, the sensitivity was 1000 copies, suggesting that this assay is a sensitive and specific method for detecting respiratory viruses.[77]

The Qiagen ResPlex II V2.0 kit, which uses a target-enriched multiplexing RT-PCR amplification combined with a suspension array detection, detects a total of 17 respiratory viruses, including Flu A/B,

TABLE 2 Diagnostic approaches for multiplex respiratory virus infections

Diagnostic approaches	Virus detected	References
NxTAG-RPP	hAdV, hRV, influenza virus	73
mOTNRT-PCR	RSV, hRV, hMPV	74
Panther fusion respiratory assay	Flu A/B, RSV, PIV, hAdV, hMPV, hRV	75
FTD21 kit	hAdV, RSV A/B, coronaviruses (229E, OC43, NL63, and HKU1), hPIV 1/2/3/4, Flu A (H1, H1-2009), Flu B, hRV, hMPV A/B	76
GeXP assay	Flu A (H1), Flu B, hPIV 1/2/3, hRV, hMPV, hAdV, RSV A/B, coronaviruses (229E, OC43, NL63, and HKU1), hBoV	77
Qiagen ResPlex II V2.0 kit	A/B, hPIV 1/2/3/4, RSV, hMPV, RV, EV, hBoV, hAdV, coronaviruses (229E, OC43, NL63 and HKU1) and FluA pdm09	78
FilmArray multiplex PCR system	hAdV, RSV A/B, coronaviruses (229E, OC43, NL63, and HKU1), hMPV, hRV/hEV, Flu A (H1, H1-2009, H3), Flu B, PIV 1/2/3/4	79

Abbreviations: Flu A/B, influenza A/B; hAdV, human adenovirus; hBoV, human bocavirus; hEV, human enterovirus; hMPV, human metapneumovirus; hRV, human rhinovirus; PIV, parainfluenza virus; RSV, respiratory syncytial virus.

PIV 1/2/3/4, RSV, hMPV, rhinoviruses (RVs), enterovirus (EV), human bocavirus (hBoV), hAdV, four coronaviruses (229E, OC43, NL63, and HKU1) and FluA pdm09 from 438 nasopharyngeal swab specimens.[78] The specificity of the test was about 92.9% to 100%. The sensitivity for PIV3, hMPV, PIV1 and BoV was reported to be 73.1%, 70%, 66.7%, and 55.6%, respectively, but the sensitivity for FluA, EnV, OC43, RSV and H1N1 was relatively low.[78]

The FilmArray multiplex PCR system is a multiplex PCR panel that can be used to detect 17 viruses, including RSV-A and RSV-B, and 2 bacteria. This platform requires 2 minutes of hands-on time for sample preparation and about 1 hour for device running time. The testing platform integrates cell lysis reagents, DNA/RNA extraction reagents, purification reagents, amplification reagents, and also detection reagents into one pouch.[79] After finishing the run, software analyses were performed, and the test results were presented in an integrated table that includes all tested pathogens.[79] The studies have revealed the sensitivity to be 86.4% for RSV-A and 100% for RSV-B, and the specificity was 100% for RSV-A and RSV-B compared to a combination reference of eSensor, xTAG, and a laboratory-developed test.[78] The sensitivity and specificity were 100% and 99.5% compared to the Prodesse assay.[79]

8 | CONCLUSIONS

Respiratory viruses are a major cause of symptomatic respiratory tract infection in all age groups worldwide. Timely and accurate diagnosis of these viruses enables appropriate treatment of infections. Traditional and modern molecular diagnostic approaches with their advantages, disadvantages and principles, are summarized for several common respiratory viruses, such as influenza virus, human RSV, coronavirus, hAdV, and rhinovirus, as discussed in this review. However, these respiratory viruses are prone to antigenic drift caused by point mutations in viral genes, and new strains with pandemic potential may result from gene reassortment. All of these continue to pose new challenges to rapid and accurate detection of respiratory virus infection in humans. Therefore, the identification of mutations within viral genomes is quite essential. However, efforts to pinpoint genetic mutations in human respiratory viruses have relied on high-throughput sequencing of single genes or gene families using Sanger sequencing. Although this approach has been fruitful, the cost and throughput of Sanger sequencing generally prohibits systematic sequencing of the ~22 000 genes that make up the exome. The recent development of NGS technologies changed this paradigm by providing the capability to rapidly sequence exomes, transcriptomes, and genomes at relatively low cost. The application of NGS in diagnosis and identification of respiratory pathogens especially for patients with severe pneumonia or those with unknown origin infections becomes more and more popular. NGS exhibited the ability to detect coinfections in severe pneumonia patients and performed well in diagnosing severe pneumonia compared to conventional methods. As an infrequently circulating genotype, genomic characterization of the whole genome of a viral strain will promote further studies on its epidemiology and pathogenicity, and aid the diagnosis of a new emerging respiratory virus. WGS enables better identification of transmission events and outbreaks, which is not always possible with sub-genomic sequences.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

NZ, LW, XD, RL, MS, CH, YS, and LH drafted the manuscript. FY, LD, and SJ revised and edited the manuscript. All authors read and made final approve of the manuscript.

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