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Christopher C. Attaway, MD, MSc
Medical Microbiology Fellow, Robert J. Tomsich Department of Pathology and Laboratory Medicine, Diagnostics Institute, Cleveland Clinic, Cleveland, OH

Hannah Wang, MD, D(ABMM)
Medical Director, Molecular Microbiology and Virology Laboratories, Robert J. Tomsich Department of Pathology and Laboratory Medicine, Diagnostics Institute, Cleveland Clinic, Cleveland, OH; Assistant Professor, Cleveland Clinic Lerner College of Medicine of Case Western Reserve University, Cleveland, OH

Laboratory testing for respiratory viruses for the clinician

ABSTRACT

Testing for respiratory viruses has changed greatly over the past decade, owing to advances in technology, drug development, vaccine research, and a growing recognition of the importance of improving patient access. Here, we focus on the most common respiratory viruses and review preanalytic variables (eg, collection and storage) that affect test results, testing methods including nucleic acid amplification testing (NAAT), and controversies, challenges, and trends in diagnostic testing relevant to clinicians.

KEY POINTS

With seasonal patterns of SARS-CoV-2, influenza A and B, and respiratory syncytial virus still in flux, testing strategies for these and other common respiratory viruses will depend on patient- and institution-specific factors.

NAAT is the most sensitive method for detecting respiratory viruses, although other types of testing may be useful in specific situations. Specimens should be collected by the clinician using nasopharyngeal flocked swabs and transported to the laboratory in viral transport medium.

Expanded multiplex NAAT panels for respiratory pathogen detection have become increasingly popular, but their cost-effectiveness and clinical utility outside of immunocompromised populations remain unknown.

No current diagnostic tests can reliably predict whether a NAAT-positive patient is still infectious or rule out a detected respiratory virus as the cause of a patient's symptoms.

Home-collection and over-the-counter home testing for respiratory viruses are likely to grow, bringing challenges and opportunities to both laboratorians and clinicians.

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Acute respiratory illnesses—most of which are caused by viruses—have always imposed a burden on individuals, the healthcare system, and society. The COVID-19 pandemic demonstrated this on a scale not seen in recent history, affecting how we understand, diagnose, study, treat, and prevent respiratory viral illness. Two newly available US Food and Drug Administration (FDA)-approved vaccines for respiratory syncytial virus (RSV) for older adults and pregnant women have added to public awareness of respiratory infectious diseases beyond the well-known influenza viruses.

The testing landscape for acute viral respiratory illnesses is much different today than it was even 5 years ago, owing to technological advances in testing, new treatments and vaccines, and a growing recognition of the importance of improving patient access. Here, we provide an overview of the most common viral respiratory illnesses, describe how and when to test for them, and highlight important trends and controversies in diagnostic testing.

THE USUAL CULPRITS

In the wake of the COVID-19 pandemic, the 3 respiratory viruses causing the most deaths and illnesses are SARS-CoV-2, influenza viruses, and RSV. As the seasonal winter pattern of influenza and RSV infections has approached the pre-COVID-19 baseline, we are faced with the possibility of continued "tripledemic" winters in which all 3 viruses circulate simultaneously.

Although influenza and RSV circulation were suppressed from 2020 through 2022, they returned to nearly prepandemic levels in the 2022–2023 winter season. In 2022–2023, influenza A and B were responsible for an estimated 31 million symptomatic infections, 14 million healthcare visits, 360,000 hospitalizations, and 21,000 deaths in the United States.[1] RSV is less common but still important, accounting for 58,000 to 80,000 hospitalizations and 100 to 300

deaths in children younger than 5 years, and an estimated 60,000 to 160,000 hospitalizations and 6,000 to 10,000 deaths in older adults.[2] SARS-CoV-2, the agent causing COVID-19, is transitioning to a winter-predominant seasonal pattern as well, although it continues to circulate year-round at a baseline level. Patients infected with any of these viruses can present with overlapping symptoms, making diagnostic testing necessary so that patients receive the correct targeted therapy.

Influenza, RSV, and SARS-CoV-2 can cause similar systemic symptoms including fever, chills, and headaches, and can infect the lower respiratory tract.[3] Therapeutic or preventive interventions are now available for each.

Other respiratory viruses such as adenoviruses, rhinoviruses, human metapneumovirus, and parainfluenza viruses 1–4 also can present with similar symptoms (**Table 1**).[4–6] Bocaviruses have also been associated with respiratory illnesses, particularly in children, but their etiologic role is controversial because they are frequently detected in people without symptoms and in co-infections.[7] Currently, no targeted therapies or vaccines are available for these other viruses; however, diagnostic testing can be useful in immunocompromised and severely ill patients to focus the differential diagnosis and guide other aspects of care.[8] Other viruses such as cytomegalovirus, herpes simplex viruses, varicella zoster virus, non-rhinovirus enteroviruses, parechoviruses, hantaviruses, Middle Eastern respiratory syndrome virus, and measles morbillivirus may also cause respiratory illness in certain populations but are not discussed here.

Below, we review diagnostic testing for the most common respiratory viruses, focusing on nucleic acid amplification tests (NAATs).

TRASH IN, TRASH OUT: PREANALYTIC VARIABLES

Before a test is even run, how it is collected, transported, stored, and processed can affect its accuracy. Optimum conditions for specimen collection and transport allow accurate downstream testing without delays. Specimen type must be considered, as well as the type of swab and the transport media.

Where to sample? Respiratory viruses infect and replicate in the ciliated respiratory epithelium of the upper respiratory tract—especially the posterior nasopharynx but also the oropharynx and anterior nares—and can be found in saliva. To varying degrees, they can also infect the lower respiratory tract; some patients may present with lower respiratory tract disease, which can be missed by sampling only the upper respiratory tract.

Consistently, nasopharyngeal swabs have the best sensitivity (generally 90% to 100% depending on virus and test platform) for viral NAAT among upper respiratory specimens.[9,10] By comparison, testing of saliva has a lower and more variable sensitivity (90.8% vs 96.1% in one study).[11] Nasal and oropharyngeal (throat) swabs are less favorable; a meta-analysis[12] found the following sensitivities:

- Nasal swabs 82% (95% confidence interval [CI] 73%–90%)
- Throat swabs 84% (95% CI 57%–100%)
- Saliva samples 88% (95% CI 81%–93%).

Testing of bronchoalveolar lavage fluid, sputum, and endotracheal aspirate for lower respiratory tract infections has a sensitivity exceeding 80% in patients with pneumonia.[13] Importantly, lower respiratory tract specimens can be positive in approximately 7% of cases in which upper respiratory tract specimens tested negative.[14]

What type of swab? Upper respiratory tract specimens are best accessed with a swab, but not all swabs are acceptable for NAATs. Flocked swabs, composed of rows of perpendicular synthetic fibers, are ideal because they have more surface area and are better at releasing pathogens from the swab into a liquid medium for recovery.[15] Traditional spun fiber, synthetic (rayon, polyester), or organic (cotton) swabs demonstrate inferior sensitivity compared with flocked swabs. Swabs with wooden shafts, which may contain formaldehyde or calcium alginate, decrease virus recovery and interfere with nucleic acid amplification,[16] and some laboratories reject them.

What type of transport medium? Most FDA-cleared NAATs are approved for specimens collected with a flocked swab and placed in a liquid medium such as viral transport medium or universal transport medium.[17] These media consist of buffered salt solutions with protein-stabilizing agents and include antimicrobials to prevent bacterial and fungal overgrowth.

Dry swabs (specimens collected and placed into a container without transport medium) need to be rehydrated upon receipt but have the advantages of lightweight transportation and less risk of spilling during shipping and storage. For SARS-CoV-2, specimens obtained with dry swabs have recovery rates comparable with those of specimens received in transport medium, but medium is preferred for ideal specimen integrity.[18] For influenza, one study showed that using dry swabs at ambient temperature resulted in a lower detection rate of influenza, but no differ-

TABLE 1
Viral causes of common respiratory syndromes

	Strength of association with clinical syndrome				
Virus	Common cold	Croup	Bronchiolitis	Influenza-like illness	Pneumonia
Influenza A or B	Moderate	Weak	Weak	Strong	Strong
Respiratory syncytial virus	Moderate	Moderate	Strong	Moderate	Strong
SARS-CoV-2	Moderate	Weak	Negligible	Strong	Strong
Seasonal coronaviruses[a]	Moderate	Weak	Weak	Weak	Weak
Rhinovirus	Strong	Weak	Negligible	Weak	Moderate
Human metapneumovirus	Moderate	Weak	Strong	Weak	Moderate
Parainfluenza viruses 1–4	Moderate	Strong	Moderate	Weak	Moderate
Adenoviruses	Moderate	Weak	Negligible	Moderate	Strong

[a]Coronaviruses 229E, HKU1, NL64, or OC43

Data from references 4–6

ence was seen when specimens were refrigerated.[19] In the event of supply chain shortages, as in the initial SARS-CoV-2 pandemic, dry swabs can be an option when transport media are not available, but at a cost of lower sensitivity.

Time matters. Ideally, specimens should be transported to the laboratory for testing as quickly as possible. Depending on the desired test, it is crucial to consult the performing laboratory test directory or the laboratory itself to ensure that proper collection, storage, and transportation conditions are met to prevent specimen rejection or inaccurate results. In general, nucleic acids (and particularly RNA) are more stable at lower temperatures. Duration of specimen transport in ambient conditions, and repeated freeze-thaw cycles should be minimized for optimal assay sensitivity.

THE NEW GOLD STANDARD: NUCLEIC ACID AMPLIFICATION TESTS

NAAT has become the gold standard for diagnosing respiratory infections because it is more sensitive than other tests and can be done in quantity and quickly. The most used NAAT method is real-time reverse transcription polymerase chain reaction (PCR), which detects an increasing fluorescent signal as the target is amplified during cycles of heating and cooling.[20] If the fluorescence value exceeds a preset baseline, the specimen is deemed positive for the given target. Fluorescence is measured after each PCR cycle, and the cycle number at which the fluorescent signal rises above baseline is called the cycle threshold value.

Not all amplification techniques produce a cycle threshold value. Some assays use end-point PCR, which measures fluorescence only at the end of all the cycles. Additional isothermal methods, such as loop-mediated amplification or transcription-mediated amplification, amplify the target without the temperature cycling steps used in PCR.[21]

Testing for multiple viruses at once

Nucleic acid testing can be performed as a single-target test (singleplex), which was more useful early in the COVID-19 pandemic when SARS-CoV-2 was the only circulating respiratory virus and isolation strategies and travel regulations were test-based. Limited multiplex panels (with 3 to 5 pathogen targets) are now commercially available for SARS-CoV-2, influenza A and B, and RSV and are expected to become more useful, given the expected seasonal cocirculation of all 3 viruses.[17] Limited multiplex testing can often be achieved in a single reaction through use of different fluorophores for each viral target, reducing reagent and labor costs.

Expanded multiplex syndromic panels (with more than 5 pathogen targets) that include viruses and atypical bacteria are available and can often distinguish between viral subtypes [ie, influenza A(H1N1pdm09) vs A(H3N2)].[22] These expanded panels often use technologies such as microfluidics, microelectronics, or labeled beads to achieve high-

level multiplexing. These advanced technologies can have higher reagent costs, however.

Testing with singleplex or limited multiplex panels is ideal for most adult outpatients with symptoms, especially considering the cost of expanded multiplex panels.[23] Testing with these systems can be performed by large, centralized laboratories; some smaller platforms can be used in urgent care clinics and emergency departments to aid in patient triage. In comparing singleplex assays with their expanded panel counterparts, stand-alone PCR assays overall perform better for influenza and RSV, although overall sensitivity for multiplex panels ranges from 80% to greater than 99%, depending on the pathogen.[24,25] Previously, influenza and RSV NAATs were routinely offered only during high-prevalence winter months, but with the COVID-19 pandemic disrupting seasonal trends, many laboratories are now offering influenza and RSV NAATs year-round.

Expanded multiplex panel testing, with as many as 33 pathogen targets, has been associated with reduced hospital admissions, shorter hospital lengths of stay, and decreased antibiotic use.[26] In most studies, however, the effect of expanded panels on hospital length of stay and antibiotic use was limited to influenza.[27–31] In fact, point-of-care tests for influenza, either antigen- or nucleic acid-based, are the biggest drivers in reducing hospital admissions and unwarranted antibiotic use.[32]

Many factors that are difficult to measure affect decisions about whether to admit patients to the hospital and how to treat them, and assessing the results of expanded multiplex testing within that scope is challenging.[33] These expanded multiplex panels may offer a result that is not clinically actionable but does give the patient and their providers peace of mind; however, the cost of such testing can range from hundreds to thousands of dollars.[34] Multiplex panels should be considered in immunocompromised or severely ill patients when a limited panel is negative but clinical suspicion remains high for viral illness.

Disadvantages of NAAT

Although respiratory viral testing by NAAT has the many advantages described above, it also has some disadvantages. Some viruses are genetically similar but cause different clinical syndromes, so that cross-reactivity is a problem. For example, most respiratory viral panels cannot distinguish between rhinoviruses and enteroviruses.

Another limitation is that as respiratory viruses evolve, they can develop mutations in primer or probe binding sites, leading to loss of assay sensitivity.[35]

One of the biggest limitations of NAAT is that not all positive results may correlate with active symptomatic infection, which is discussed below.

Lastly, expanded multiplex panels come with their own sets of limitations that can create challenges in result interpretation. Co-infections are not uncommon, and the probability of at least 1 false-positive result on a large multiplex panel inevitably is statistically higher than with more limited testing.[10] These panels cover the most common viruses—but not every viral cause of respiratory illness, and more importantly, most cannot rule out superimposed bacterial infection.

ANTIGEN DETECTION TESTS

Viral proteins can be detected with antigen tests such as direct fluorescent antibody or lateral flow assays (rapid antigen tests).

Direct fluorescent antibody tests require interpretation by fluorescent microscopy or a molecular analysis platform; they are available for many viruses and have a higher sensitivity than rapid antigen tests. They typically produce results in 2 to 4 hours.[36]

Rapid antigen tests are available for SARS-CoV-2, influenza, and RSV but are not commonly used for other viruses. For example, rhinoviruses, which cause the common cold, are a large, heterogenous group that lack a common target for reliable antigen detection.[37]

Rapid antigen tests are ideal for use in outpatient clinics and urgent care facilities, and some current tests can also be used in nursing homes, daycare centers, schools, or at home.[25] Many of these tests are Clinical Laboratory Improvement Amendments-waived (ie, deemed so simple that there is little risk of error and therefore can be performed at sites with fewer regulatory requirements), are easy to use, and can help guide clinical management; however, they are not intended for high-throughput testing (unlike most NAATs).[25]

Although rapid antigen tests are convenient and generally have high specificity, most have substantially lower sensitivity than NAATs, typically 50% to 80% for SARS-CoV-2.[38] The FDA requires that antigen-based rapid influenza diagnostic tests have a minimum sensitivity of 80%, which is supported by independent studies.[39] These tests perform better when the prevalence is high and patients have symptoms, but a negative test cannot rule out an infection. For SARS-CoV-2, serial antigen testing is recommended to reduce the risk of false-negative results. For both influenza and SARS-CoV-2, a positive

test can lead to prescription for outpatient antiviral therapy, thereby reducing antibiotic use and further ancillary testing.[40]

Although most discourse about antigen testing centers on respiratory specimens, several studies have shown the potential value of quantitative serum or plasma SARS-CoV-2 antigen testing as a marker for predicting severe or protracted COVID-19 disease.[41] This testing is not yet available clinically—prospective studies will be required to determine the utility of antigenemia in immunocompromised patients with complex clinical presentations.

VIRAL CULTURE

Traditional viral culture is technically challenging, takes a long time to do, poses risks to laboratory personnel, and requires a biosafety level 3 facility for some viruses.[42] Even faster methodologies such as shell vial culture typically take 2 to 7 days.

For routine diagnosis, viral culture has been supplanted by NAATs because of its inferior sensitivity and longer turnaround time. However, viral culture still plays an important role in virus discovery and characterization and serves as the standard to which other testing methods and antiviral drugs are held.[43] Current guidelines do not recommend viral culture for routine respiratory viral diagnosis, reserving it for epidemiologic studies.[44]

SEROLOGIC TESTING

Antibody detection (serologic testing) is not useful for diagnosing acute respiratory viral illnesses because they are seasonal, and many people are exposed. Serologic testing cannot differentiate between prior and active infection. It may however be helpful as a way to assess the response to vaccination in patients who are immunosuppressed, or to determine whether a patient is seronegative for SARS-CoV-2 and should be given convalescent plasma.[45,46]

SEQUENCING-BASED TESTING

Metagenomic next-generation sequencing has been used in research and public health settings to discover new or uncommon viruses causing respiratory disease, but it is not yet clinically available, likely because it is expensive and has low diagnostic yield in routine settings.[43]

Viral whole-genome sequencing is similarly unavailable clinically but has been helpful in epidemiologic surveillance. Indeed, before the Omicron variant became dominant in the COVID-19 pandemic, variant subtyping helped direct treatment recommendations and predict the utility of various monoclonal antibody therapies.[47] In rare cases of treatment-resistant influenza, particularly in immunocompromised patients and young children, specimens can be sent for sequencing (and possibly phenotypic assays) at some public health laboratories to test for drug resistance.

TESTING FOR INFECTIVITY

A primary complaint concerning NAATs for respiratory viruses is that they can detect genomic material for weeks or months after symptoms resolve. Consequently, the results can be challenging to interpret for patients with complex cases with serial presentations, and prolonged NAAT positivity can complicate infection prevention and isolation workflows. Unfortunately, no test can reliably predict whether a NAAT-positive patient is still infectious or rule out a detected respiratory virus as the cause of a patient's symptoms.[48]

Some have advocated using viral culture as a surrogate for infectivity. Although growth in culture does indicate that a virus is present that can replicate, a negative culture does not rule out infectivity, because viral culture has relatively low clinical sensitivity. A household transmission study of SARS-CoV-2 during 2020–2022 found that 6 (21%) of 29 household contacts of primary patients who were culture-negative were infected with SARS-CoV-2.[49]

Others have advocated for use of viral load, or its imperfect surrogate, the cycle threshold value, as a measure of infectivity or disease activity. Although low viral load (as indicated by a higher cycle threshold value) correlates with inability to culture virus, a 2020 to 2021 study of SARS-CoV-2 demonstrated no relationship between initial viral load and likelihood of transmission to household contacts.[50]

Cycle threshold values are even more fraught with interpretive risk, as they can vary by as many as 3 cycles on the same PCR instrument, and up to 12 cycles between different assay platforms.[51] A systematic review of 33 studies of respiratory viruses, including SARS-CoV-2, found no conclusive correlations between cycle threshold value and clinical outcomes.[52]

More data are needed on serial antigen testing and viral subgenomic RNA intermediate testing as markers of infectivity; most studies of these tests have used viral culture as a surrogate for infectivity, which is an imperfect measure as noted above.

Patient history, immune status, testing platforms,

gene targets, and risk tolerance must be considered when interpreting NAAT results for infectivity. Cycle threshold values and viral loads from quantitative SARS-CoV-2 testing may not be particularly useful in isolation but may be helpful when a single assay is repeated serially for the same patient. Consultation with the microbiology laboratory's medical director may be helpful. As COVID-19 severity declines with increasing population immunity, symptom-based or time-based isolation exit strategies will likely replace test-based strategies.

INCREASING CONVENIENCE AND ACCESS: DECENTRALIZED SPECIMEN COLLECTION AND TESTING

With its potential to expand access to laboratory, public health, and overall healthcare services, at-home patient collection of specimens has become a focus for respiratory testing.[53] In 2022, under the FDA Emergency Use Authorization (EUA), testing of specimens collected by the patient at home became available for SARS-CoV-2, influenza A and B, and RSV. Test kits do not require a prescription, and patients collect the specimen themselves and mail or return it in designated drop boxes to the performing laboratory. In a feasibility and performance study, patient-collected saliva specimens yielded results comparable to those of physician-collected nasal swab specimens for detecting SARS-CoV-2 by PCR.[54]

Over-the-counter (OTC) rapid antigen tests for SARS-CoV-2 also have EUA.[55] Unlike influenza rapid testing, SARS-CoV-2 home antigen tests do not have minimum sensitivity standards. Depending on the manufacturer, test sensitivity ranges from 30% to more than 95%; they perform particularly poorly when patients have no symptoms.[56] To increase test sensitivity, at-home tests for SARS-CoV-2 are intended to be repeated when initially negative. However, individuals do not always follow this guidance. The first SARS-CoV-2 rapid antigen test to be fully cleared by the FDA for OTC home use in November 2023 demonstrated 89.8% sensitivity in people with symptoms but only 27.5% in those without symptoms.[56,57] The Lucira by Pfizer Check-It COVID-19 Test, a patient-collected and performed test kit, is a NAAT that uses an isothermal amplification technique and has greater than 90% sensitivity.[58]

Multiplex OTC at-home respiratory virus tests are starting to be developed, with the first (Lucira by Pfizer COVID-19 & Flu Test) receiving FDA EUA for SARS-CoV-2 and influenza A and B in September 2023.[55] At-home testing for other viral target combinations is available internationally.[59]

The COVID-19 pandemic brought to the fore some important limitations of laboratory-based testing, including lack of access and equity, exposure considerations during specimen collection, high cost, suboptimal turnaround times, and supply-chain failures. Clinician-collected laboratory-based testing for respiratory viruses remains the gold standard, but decentralized testing and specimen collection fill an important gap in caring for patients with respiratory viral infections. The medical and public health communities will need to address the challenges this type of testing poses in terms of quality management, patient education, and epidemiologic monitoring.

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Correspondence: *Hannah Wang, MD, D(ABMM), Medical Director, Molecular Microbiology and Virology, Department of Laboratory Medicine, LL1-2, Cleveland Clinic, 9500 Euclid Avenue, Cleveland, OH 44195; wangh7@ccf.org*