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MINIREVIEW

Detection of Influenza A and B Viruses and Respiratory Syncytial Virus by Use of Clinical Laboratory Improvement Amendments of 1988 (CLIA)-Waived Point-of-Care Assays: a Paradigm Shift to Molecular Tests

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ABSTRACT An accurate laboratory diagnosis of influenza, respiratory syncytial virus (RSV), and other respiratory viruses can help to guide patient management, antiviral therapy, infection prevention strategies, and epidemiologic monitoring. Influenza has been the primary driver of rapid laboratory testing due to its morbidity and mortality across all ages, the availability of antiviral therapy, which must be given early to have an effect, and the constant threat of new pandemic strains. Over the past 30 years, there has been an evolution in viral diagnostic testing, from viral culture to rapid antigen detection, and more recently, to highly sensitive nucleic acid amplification tests (NAAT), as well as a trend to testing at the point of care (POC). Simple rapid antigen immunoassays have long been the mainstay for POC testing for influenza A and B viruses and respiratory syncytial virus (RSV) but have been faulted for low sensitivity. In 2015, the first POC NAAT for the detection of influenza was approved by the Food and Drug Administration (FDA), ushering in a new era. In 2017, the FDA reclassified rapid influenza diagnostic tests (RIDTs) from class I to class II devices with new minimum performance standards and a requirement for annual reactivity testing. Consequently, many previously available RIDTs can no longer be purchased in the United States. In this review, recent developments in Clinical Laboratory Improvement Amendments of 1988 (CLIA)-waived testing for respiratory virus infections will be presented, with the focus on currently available FDA-cleared rapid antigen and molecular tests primarily for influenza A and B viruses and RSV.

KEYWORDS CLIA waived, NAAT, PCR, RIDT, influenza, point of care, rapid antigen test, respiratory syncytial virus, respiratory viruses, viral diagnosis

The global burden of respiratory virus disease is substantial. The World Health Organization (WHO) estimates that 3.9 million people succumb to acute respiratory viral infections every year (1). Respiratory viral infections increase the risk of secondary bacterial infections, including pneumonia and sepsis, and trigger more than half of asthma and chronic obstructive pulmonary disorder exacerbations. In children below the age of 5, the combined global mortality of influenza and respiratory syncytial virus (RSV) approaches 300,000 deaths per year (1). Other respiratory viruses, including adenoviruses, parainfluenza virus (PIV) types 1 to 4, human metapneumovirus (HMPV), human coronaviruses (HCoV), and rhinoviruses (RV), are associated with lower mortality but significant morbidity. Respiratory viral infections also impose a substantial economic burden (2, 3).

Since signs and symptoms can overlap, a clinical diagnosis is not sufficient to differentiate infections with various viral and bacterial pathogens. A rapid and accurate

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viral diagnosis can reduce the overuse of antibiotics and enable prompt administration of antiviral therapy. In addition, a prompt viral diagnosis can facilitate correct infection prevention practices, enable shorter hospital stays, and assist epidemiologic monitoring for public health.

Over the past several decades, viral diagnostic methods have evolved to provide more rapid results, from conventional viral culture to rapid centrifugation cultures, direct fluorescent antibody (DFA) and rapid antigen immunoassays, and most recently, to highly sensitive molecular amplification methods. Currently, the need for more accessible testing to aid clinical decision making is driving an expansion of point-of-care (POC) testing (4). Many tests used in the POC setting in the United States have received a Clinical Laboratory Improvement Amendments of 1988 (CLIA) waiver. CLIA-waived tests employ low complexity methodologies with minimal potential for errors and use unprocessed specimens that require no operator manipulation. Consequently, waived tests can be performed by nonlaboratory personnel in physicians' offices and various other POC locations once a CLIA Certificate of Waiver has been obtained (5).

While CLIA-waived lateral flow immunoassays have been a mainstay of POC testing for influenza virus and RSV for years, they have lacked sensitivity (6, 7). Then in 2015, the first CLIA-waived nucleic acid amplification test (NAAT) for the detection of influenza virus was cleared by the Food and Drug Administration (FDA), ushering in a paradigm shift in POC testing. In another potentially transformative change, in 2018 the FDA implemented more-stringent performance requirements for rapid influenza immunoassays (8). In this review, developments in CLIA-waived POC testing for influenza A and B viruses and RSV and other respiratory viruses will be presented, with the focus on currently available FDA-cleared rapid antigen tests and emerging molecular technologies.

SAMPLE COLLECTION, VIRAL LOAD, AND TEST PERFORMANCE

Before comparing test methods, including published studies, it should be emphasized that regardless of the method, test performance is highly impacted by the viral load in the specimen (9–13). Thus, studies that include a high proportion of samples from young children routinely report superior test performance that is not replicated when older adults comprise the majority of the subjects. However, regardless of patient age, the viral load can be enhanced when samples are collected 24 to 72 h after symptom onset, when viral shedding is maximal. Nasopharyngeal (NP) specimens for influenza collected either less than 1 day or more than 4 days after symptom onset have been associated with lower sensitivity (14). In addition, some influenza virus strains have been associated with lower virus shedding, which in turn lowers their detection rate (15). Other variables impacting viral load include the skill of the specimen collector, the use of flocked swabs, the volume of viral transport medium (VTM) used, and the type of sample collected. NP aspirates (NPA) and nasal washes (NW), long considered the samples of choice, have largely been supplanted by NP swabs (NPS) due to the convenience and ease of use. Some test kits utilize a nasal swab (NS), which is more comfortable for the patient. For Alere i influenza A & B NAAT kits, a special foam swab is provided, which is inserted into the anterior nares to absorb secretions. It is then placed in a paper sleeve, rather than diluted in transport medium, prior to testing. It should be noted that for CLIA-waived tests, the approved sample types can differ from the nonwaived versions of the tests and are specified in the manufacturer's instructions, which must be strictly followed in their entirety to maintain waived status.

ANTIGEN DETECTION IMMUNOASSAYS FOR INFLUENZA AND RSV

Current antigen detection immunoassays do not amplify either the target or the signal and are therefore inherently less sensitive than culture or nucleic acid amplification tests. Rapid immunoassays for respiratory viruses are limited to influenza A and B viruses and RSV, which are sold as separate kits. While influenza A and B tests are used in all age groups, rapid RSV antigen tests are more commonly used in pediatric populations (7).

Description of methods. The most frequently used POC antigen detection method is the lateral flow immunochromatography or strip test (Table 1). A specified amount of sample is simply added to the test strip, the timer is set for 10 to 15 min, according to kit instructions, and the result is read promptly at completion. In the sandwich assay format, the target viral analyte contained in a liquid clinical sample is transported laterally on a horizontal nitrocellulose membrane by capillary flow, bound by chromophore-labeled antibodies (usually colloidal gold), and then immobilized by a line of membrane-bound antibodies, producing a visible capture zone or test line. A control line is produced when a nontarget analyte in the sample is captured by immobilized antibodies, indicating a valid test. Dipstick chromatography is a variation of the lateral flow assay in which the test membrane is vertically dipped into a clinical sample in buffer.

To improve the sensitivity and specificity, two commercially available lateral flow tests use an instrument-based digital scan of the test strip to enhance the detection of virus antigens. One uses a reflectance-based measurement of modified colloidal metal particles to evaluate the line signal intensities on the assay test strip (BD Veritor; Becton Dickinson Diagnostics, Sparks, MD, USA). In the other, europium-based fluorescent-labeled antibodies are substituted for chromophores, enabling fluorometric detection by a small automated strip reader (Sofia; Quidel Corp., San Diego, CA, USA). These digital immunoassays (DIAs) provide several advantages over visual readouts, including greater sensitivity, a reduction in interoperator variability, objective results, the ability to scan the identifications of the operator, patient and kit, a result printout, and the potential to interface with the laboratory information system (LIS) (16). Newer versions of the instruments, Becton Dickinson BD Veritor Plus and Quidel Sofia 2, have more features than the original instruments, as discussed below (see "Current CLIA-Waived Rapid Antigen Tests").

Past rapid antigen test performance. Past studies by the Centers for Disease Control and Prevention (CDC) have shown that positive lateral flow rapid influenza diagnostic test (RIDT) results typically required 10^5 to 10^6 infectious viral particles, and their performance varied with circulating viral strains (9). Although the manufacturers' product inserts have commonly listed sensitivities of 82 to 99% compared to that in cell culture in package inserts, the sensitivity in published studies has averaged 54 to 61% compared to that for NAAT (16–18). While the specificity has been consistently high, the CDC recommends that RIDTs not be used when the viral prevalence in the community is low, when false-positive results are more likely (https://www.cdc.gov/flu/professionals/diagnosis/clinician_guidance_ridt.htm). Studies have consistently shown that DIAs outperform visually read RIDTs (10, 11, 19); however Quidel Sofia was found by a number of investigators to have a significant number of false-positive influenza B results that subsequently led to a reagent recall by the company (16, 19).

The sensitivities of CLIA-waived RSV rapid antigen diagnostic tests (RADTs) in the published literature have averaged 75 to 80% (7, 17). The assay sensitivity is superior in young children less than 2 years of age, due to higher viral shedding. In contrast, in patients over 10 years of age, the sensitivity can be lower by 13 to 65 percentage points (20, 21). For DIAs, the results for BD Veritor and Quidel Sofia are consistently positive for samples with higher viral loads, but lower viral loads are missed (22). Better results can be obtained when samples are collected within 2 days of symptom onset and with NP as opposed to nasal swabs.

Recently, several systematic reviews and meta-analyses of influenza and RSV rapid antigen tests have been published. The review by Merckx et al. in 2017 focused on influenza alone and included 162 studies, 130 of RIDTs, 19 of DIAs, and 13 of NAATs (6). The pooled sensitivities for influenza A virus detection were 54.4% for RIDTs, 80.0% for DIAs, and 91.6% for NAATs. For influenza B virus, the sensitivities were 53.2% for RIDTs, 76.8% for DIAs, and 95.4% for NAATs. The pooled specificities were >98%. The pooled sensitivities for studies in children were higher by 12.1 to 31.8 percentage points for immunoassays. The authors noted incomplete reporting of factors that could increase

TABLE 1 CLIA-waived rapid antigen tests for influenza A & B and RSV[a]

Kit	Manufacturer	Method	Viral target	Specimens[c]	Instrument[d]	Incubation (min)	Comments
Influenza[b]							
Alere influenza A & B test	Alere, Scarborough, ME, USA	Dipstick immunochromatography	Influenza A & B nucleoproteins	NS	No	10	Uses direct nasal swab
BD Veritor system for rapid detection of Flu A+B	Becton, Dickinson, Sparks, MD, USA	Digital immunoassay	Influenza A & B nucleoproteins	NS, NPS	BD Veritor and BD Veritor Plus Reader	10	Incubate on bench. Insert into reader for 1 min. BD Veritor Plus has both "analyze now" and "walk away" modes
BinaxNOW influenza A&B card 2	Alere, Scarborough, ME, USA	Lateral flow immunochromatography	Influenza A & B nucleoproteins	NS, NPS, NA, NW	Alere Reader	15	New 2nd-generation card test with reader
BioSign Flu A+B[e]	Princeton Biomeditech, Princeton, NJ, USA	Lateral flow immunochromatography	Influenza A & B nucleoproteins	NS, NPS	No	10–15	Marketed under many different names
QuickVue influenza A+B	Quidel, San Diego, CA, USA	Dipstick immunochromatography	Influenza A & B nucleoproteins	NS, NPS, NA, NW	No	10	Uses direct swab without VTM
Sofia influenza A+B FIA	Quidel, San Diego, CA, USA	Fluorescence (digital) immunoassay	Influenza A & B nucleoproteins	NS, NPS, NA	Sofia and Sofia 2 analyzers	15	Incubate and read in reader in "walk away" mode. Sofia 2 has additional options to incubate on bench and then insert into reader for 1 min in "read now" mode
RSV[f]							
BD Veritor RSV	Becton, Dickinson, Sparks, MD, USA	Digital immunoassay	RSV fusion protein	NPS	BD Veritor and BD Veritor Plus Reader	10	Incubate on bench. Insert into reader for 1 min. BD Veritor Plus has both "analyze now" and "walk away" modes
BinaxNOW RSV card	Alere, Scarborough, ME, USA	Lateral flow immunochromatography	RSV fusion protein	NPS, NW	No	15	
ClearView RSV rapid test	Alere, Scarborough, ME, USA	Lateral flow immunochromatography	Not stated	NPS, NA	No	15	
QuickVue RSV	Quidel, San Diego, CA, USA	Dipstick immunochromatography	RSV fusion protein	NPS, NA, NW	No	15	
SAS RSV alert	SA Scientific Inc., San Antonio, TX, USA	Lateral flow immunochromatography	Not stated	NS, NA, NW	No	15	
Sofia RSV FIA	Quidel, San Diego, CA, USA	Fluorescence (digital) immunoassay	RSV nucleoprotein	NPS, NA, NW	Sofia and Sofia 2 analyzers	15	Incubate and read in reader in "walk away" mode. Sofia 2 has additional options to incubate on bench and then insert into reader for 1 min in "read now" mode
Sure-Vue RSV	Fisher Scientific, Pittsburgh, PA, USA	Lateral flow immunochromatography	Not stated	NPS, NA, NW	No	15	

[a]Information from package inserts and manufacturers' websites.
[b]For all influenza rapid antigen tests listed, manufacturers obtained a new 510(k) clearance and demonstrated compliance with the special controls listed in Tables 2 and 3.
[c]Some antigen kits require use of swabs provided in kit; use of small volumes of viral transport medium will enhance sensitivity. NS, nasal swab; NPS, nasopharyngeal swab; NA, nasal aspirate; NW, nasal wash.
[d]All instruments accommodate one sample at a time.
[e]Kits with different names distributed under same 510(k) number: Status Flu A&B (LifeSign, LLC), OSOM Ultra Flu A&B test (Sekisui Diagnostics, LLC), OraSure QuickFlu rapid A+B test, Polymedco Poly stat Flu A&B test, LABSCO Advantage Flu A&B, Meridian BioScience ImmunoCard STAT Flu A&B, McKesson Consult Diagnostics Influenza A&B.
[f]RSV, respiratory syncytial virus.

TABLE 2 FDA device reclassification[a]

Device	Special controls
Class II	1. Minimum clinical performance criteria 2. Annual reactivity testing and results reporting[b] 3. A provision for testing in a declared or potential emergency once samples available

[a]The FDA notified the public of the intent to reclassify RIDTs on 12 January 2017, with compliance enforced beginning 12 January 2018. Federal register, vol. 82, no. 8, Thursday, 12 January 2017, rules and regulations.
[b]Annual and emergency (new strains) analytical reactivity testing is required to monitor performance, using a characterized and updated influenza virus panel prepared by the CDC.

the risk of bias and that industry-sponsored studies reported more favorable sensitivities by 6.2 to 34.0 percentage points. Chartrand et al. (7) reviewed 71 studies on RSV RADTs, with 83% of studies conducted in children and only 3% focused on adults. They reported that the pooled sensitivity and specificity of RSV RADTs were 80% and 97%, respectively, with sensitivities higher in children (81%) than in adults (29%). The test sensitivity was lowest when PCR was used as the reference standard (74%) and highest when immunofluorescence was used (88%). Industry-sponsored studies reported significantly higher sensitivities (87% versus 78%).

Food and Drug Administration reclassification of RIDTS. When the 2009 influenza pandemic struck, many hospitals used RIDTs as their sole means of diagnosing influenza infections. It quickly became apparent that the sensitivity was suboptimal (18). As a consequence of the prominent role for influenza testing in clinical decision making and infection control initiatives, and the negative impact of antigenic variation and emergence of novel strains on testing performance, the FDA reclassified these immunoassays in 2017 from class I devices (low likelihood of harm) with general controls to class II devices (moderate likelihood of harm) with special controls (8). The class change imposes clear performance standards for sensitivity (80% versus NAAT and 80 to 90% versus culture) and specificity (95%) and a requirement to report test performance annually against circulating strains and if a new pandemic strain emerges. The details of the special controls are provided in Tables 2 and 3.

Many commonly used RIDTs did not meet the new performance standards and can no longer be sold in the United States. However, inventory purchased prior to 12 January 2018 can still be used. The new performance standards apply only to rapid immunoassays for influenza and not for RSV.

Current CLIA-waived rapid antigen tests. The waived immunoassays for influenza A and B viruses and RSV purported to meet the new standards are shown in Table 1. There are currently no CLIA-waived immunoassays for other respiratory viruses available in the United States. The two DIAs, Becton Dickinson BD Veritor and Quidel Sofia, are still available, as they meet the new performance standards. Operationally, Becton Dickinson BD Veritor provides results more quickly than Quidel Sofia, and with less hands-on time. Quidel Sofia requires an additional step, the addition of reagent buffer to dissolve the contents of the reagent tube, and both the incubation and reading times are slightly longer for Quidel Sofia (15 min and 50 s for Sofia versus 10 min and 10 s for Becton Dickinson BD Veritor [22]). The Becton Dickinson BD Veritor cassette is only inserted into the instrument for the reading step. In contrast, the Quidel Sofia requires that the 15-min incubation of the test cassette occurs within the Quidel Sofia instrument, which slows throughput. However, the Quidel Sofia 2 instrument does not

TABLE 3 Minimal performance criteria for rapid influenza immunoassays

Virus	% sensitivity (CI[a] 95% lower limit)		% specificity (CI 95% lower limit)
	NAAT[b] comparator	Culture comparator	
Influenza A	80 (≥70)	90 (≥80)	95 (≥90)
Influenza B	80 (≥70)	80 (≥70)	95 (≥90)

[a]CI, confidence interval.
[b]NAAT, nucleic acid amplification test.

have this requirement, but instead has both "analyze now" and "walk away" modes, as does the BD Veritor Plus instrument. Both can be interfaced to the LIS.

To improve sensitivity, the second-generation BinaxNOW influenza A & B card 2 test (Alere Scarborough, Inc., Scarborough, ME, USA) utilizes a small reader instead of visual reading. This test requires the use of a swab, not placed in VTM, to be inserted directly into a premeasured volume of extraction buffer in a tube, then transfer of the sample onto the test strip. QuickVue influenza A+B (Quidel, San Diego, CA, USA) uses a dipstick format and has a similar direct swab procedure but accepts washes and aspirates in addition to swabs.

The BioSign Flu A+B test (Princeton Biomeditech Corp., Princeton, NJ, USA) is sold under a variety of test names and distributors. In this test, the extraction reagent is dispensed into the extraction well of the device and the swab specimen is placed directly into the well, where it is pressed down and rotated 3 times in one direction to mix the specimen. The swab is then left in the extraction well 1 min, after which it is again rotated in one direction 3 times and then removed. The device is repositioned vertically for 1 to 2 min and then tapped and returned to the horizontal position, and the timer is started. The result is read visually at 10 to 15 min. NP aspirates and nasal washes are dispensed directly into the extraction well, followed by the extraction reagent, with the rest of the procedure as described above. A recent study of the Princeton Biomeditech BioSign RIDT, sold under the name OSOM Ultra Flu A&B test, reported results from 500 randomly selected NPS from adult patients (45% over 75 years of age) that had been previously tested by Xpert Flu PCR (Cepheid, Sunnyvale, CA, USA) in the virology laboratory (23). The RIDT was performed by the emergency department (ED) personnel after 30 min of training. The authors reported the RIDT sensitivity as 95.1% and the specificity as 98.4% compared to PCR, which is remarkable in a study confined to adults, but it is not clear whether the randomly selected study samples were limited to those with high viral loads. Further studies on the real-life performance of the current CLIA-waived tests, using prospective unselected samples, are eagerly awaited.

Advantages and limitations of rapid antigen testing at POC. The advantages of antigen detection are simplicity, low cost, speed (<15 min), and when the results are read visually, no equipment is required. Samples can be tested individually as soon as collected or batch tested. Inexpensive readers improve the sensitivity and accuracy. Since reading takes only 1 min in current instruments, it does not delay throughput. Alere BinaxNOW RSV, Quidel QuickVue influenza A+B and RSV tests, and Princeton Biomeditech BioSign Flu A+B do not use equipment and thus meet the World Health Organization (WHO) ASSURED requirements for low-resource settings (4). In general, RIDTs can also be useful for outbreak identification in nursing homes, cruise ships, schools, and other locations when multiple patients are tested.

Despite their lower sensitivity, rapid POC influenza antigen testing, by avoiding transport time to the laboratory and substantially shortening the time to result, has led to decreased ancillary testing, lower antibiotic use, and higher antiviral prescriptions in both ambulatory and inpatient settings (24, 25). It is critical that the results are available to the clinician in an actionable time frame, such as before antibiotic prescriptions are given in the outpatient setting. There are few data on the effect of RSV rapid antigen-based testing on management. In one study among hospitalized children, physicians reported that results did influence their management and the duration of antibiotics was shorter for RSV-positive children (26). In another study, RSV rapid testing facilitated the cohorting of patients in an emergency department setting (27).

NUCLEIC ACID DETECTION BY MOLECULAR AMPLIFICATION ASSAYS FOR INFLUENZA, RSV, AND OTHER VIRUSES

The 2009 influenza pandemic was key in accelerating the commercial development of user-friendly nucleic acid amplification tests (NAAT) (18). Because of its greater sensitivity, NAAT is currently considered the laboratory method of choice for the detection of respiratory viruses. Traditionally, NAAT has required multiple steps, highly

specialized technical skills, and expensive equipment and facilities, which restricted the technology to central laboratories in academic centers or reference laboratories. In recent years, some NAAT options have become streamlined into simpler and faster technologies, which involve the addition of the sample to a device and then the insertion of the device into an instrument. In January 2015, the first molecular-based test, Alere i influenza A & B, was granted a CLIA waiver by the FDA, inaugurating a new era in POC testing.

Description of methods. The current waived NAAT kits employ several methodologies, including (i) isothermal nucleic acid amplification for the Alere i influenza A and B test and Alere i RSV test (Alere Scarborough, Inc., Scarborough, ME, USA), (ii) real-time reverse transcription-PCR (rtRT-PCR) for cobas Liat influenza A/B and influenza A/B/RSV assays (Roche Molecular Systems, Pleasanton, CA, USA) and Xpert Xpress Flu or Flu/RSV (Cepheid, Sunnyvale, CA, USA), (iii) nested multiplex PCR for FilmArray respiratory panel EZ (Idaho Technology, Inc., Salt Lake City, UT, USA), and (iv) RT-PCR followed by hybridization and colorimetric visualization on a test strip for Accula Flu A/Flu B (Mesa Biotech, Inc., San Diego, CA). All require the simple addition of an unprocessed sample into a cartridge, pouch, sample receiver, or cassette, the insertion of the device into an instrument, followed by an incubation period while the reaction proceeds, with results produced in 15, 20, 30, or 60 min depending on the test and the number of analytes. Multiplex NAAT enables the simultaneous detection of multiple respiratory viruses.

The nicking enzyme nucleic acid (NEAR) isothermal amplification technique used by Alere i enables rapid amplification in a very narrow temperature range. This eliminates the need for thermal cyclers with the high-temperature DNA denaturation cycle required for PCR. The low-footprint bench-top Alere i instrument can provide 15-min results in a near-patient setting. Unfortunately, the ability of NEAR amplification to detect and discriminate between multiple targets simultaneously is limited. Thus, there is a separate Alere i RSV assay rather than a multiplexed test with influenza A and B viruses. Novel isothermal amplification methods are being developed to enhance multiplex capability (28).

In rtRT-PCR, the method used by both the Roche cobas Liat and the Cepheid Xpert Xpress, the amplification of target DNA can be monitored using emitted fluorescence as the reaction progresses, generating a positive result once a fluorescent signal crosses a specified threshold. The cycle threshold (C_T) value, defined as the number of cycles required for the fluorescent signal to cross the threshold, is inversely proportional to the viral load, with lower C_T values corresponding to higher viral loads and vice versa. In the Roche cobas Liat, the C_T value can be visualized and estimated. However, in the waived versions of Cepheid Xpert rtRT-PCR tests, the C_T value is not provided to the end user. Cepheid Xpert Xpress Flu is an improved version of the previous Cepheid Xpert Flu test. Xpert Xpress Flu has two influenza A gene targets instead of one, and the assay time has been reduced from 60 to 30 min, with positives resulted in as little as 20 min.

When multiplexed, rtRT-PCR is limited to no more than 4 to 5 targets in a single reaction vessel due to the optics of current instruments and the need for different fluorescent labels with nonoverlapping wavelengths, among other factors. Current iterations of waived tests allow for the detection of influenza A, influenza B, plus or minus RSV, and an internal control.

Nested PCR employs a two-staged amplification using outer then inner (nested) primers to enhance the detection of desired amplicons. The waived BioFire FilmArray respiratory panel EZ is a nested multiplex PCR with 14 viral and 3 bacterial targets. It employs a post-PCR melting curve analysis that differentiates targets on the basis of distinct melting temperature (T_m) peaks for each target. The results for all current BioFire FilmArray multiplex panels, including nonwaived, are qualitative only.

The Mesa Biotech Accula Flu A/Flu B assay uses multiplex RT-PCR, followed by hybridization and lateral flow technologies to provide qualitative visual detection of influenza A and B. After amplification of the sample within a test cassette inserted into the dock instrument, the amplicons are detected by hybridization with two dyed

polystyrene microsphere-conjugated oligonucleotide probes. A signal (a colored line) is generated on a detection strip, which is read visually.

The CLIA-waived Alere i, Mesa Biotech Accula, Roche cobas Liat, and BioFire FilmArray instruments accept only one sample at a time. Therefore, multiple instruments must be obtained to increase throughput. CLIA-waived Cepheid Xpert Xpress testing at POC is currently limited to two Cepheid GeneXpert instruments, GXII and GXIV, which contain two or four independent cycling modules, respectively, providing an increased capacity within one instrument. At this time, only cobas Liat and GeneXpert instruments can be interfaced with the LIS.

Current CLIA-waived molecular amplification tests. At this writing, there are eight FDA-approved molecular tests for respiratory viruses that can be performed in a POC setting (Table 4). With the exception of BioFire FilmArray, CLIA-waived NAAT POC tests target only influenza A and B viruses and/or RSV only. As with antigen tests, the sample types and transport media may vary between waived and nonwaived versions of the same test, and the manufacturers' instructions must be strictly followed to maintain waived status.

To assess the performance of CLIA-waived NAAT for their intended use, independent studies performed by nonlaboratory personnel in a POC setting would be most relevant. However, in published studies to date, the testing is more often performed in central laboratories by medical technologists. In addition, nonwaived samples and instruments are often used. For approved samples and instruments for waived testing, refer to Table 4. Published studies done in a POC setting are only available for Alere i and Roche Liat at the time of this writing.

Two published Alere i influenza A & B studies were done at POC, and only one of these used CLIA-waived direct nasal swab samples. In the first study, the comparator was cell culture, and in the second, it was PCR. When Alere i influenza A & B was compared to R-mix cell culture using direct nasal swabs from both adults and children, with testing performed at seven POC sites, the sensitivities after the resolution of discrepant results was 99.3% for influenza A virus and 96.7% for influenza B, with specificities of 98.1% and 100%, respectively (29). In contrast, when throat swabs (a nonapproved sample) from adults were tested at six POC locations in 3 hospitals in the United Kingdom using laboratory-based PCR as the comparator, Alere i had a sensitivity of 75.8% overall. Importantly, the sensitivities varied from 50 to 82% among the different POC sites in this study for unclear reasons (30). A cost analysis in this report showed the largest savings resulted from the improved accuracy of isolation for admitted patients (30).

Other studies of Alere i influenza A & B were performed in the central laboratory and used NP swabs diluted in VTM, with a laboratory-based PCR as the comparator. In one study, Alere i was 77.8% sensitive for influenza A and 85.7% sensitive for influenza B compared to rtRT-PCR, but 10- to 100-fold more sensitive than rapid antigen detection (11). In another study, the majority of the patients were children, and sensitivities of 91.4% for influenza A virus, but only 54.5% for influenza B virus, were reported (10). The poor results for influenza B were attributed to low sample numbers and low viral loads in those samples. While an effect of Alere i testing was observed on isolation practices, antibiotics, antivirals, and hospitalization rates, the authors in the latter study noted that the Alere i cost was three times that of the Quidel Sofia. Thus, they recommended that the more expensive NAAT should be used for critical populations, such as older adults at risk for complications and in whom antigen-based testing sensitivity is lower. Furthermore, to obtain the greatest benefit, rapid NAAT results should be directly linked to specific isolation precautions and to patient management algorithms, such as initiating antiviral therapy, limiting antibiotics, and reducing ancillary tests when appropriate (10, 30).

In subsequent laboratory-based studies, the Alere i influenza A & B assay was compared to another CLIA-waived NAAT (Roche cobas Liat), as well as a reference PCR. Using NPS in VTM from adults, Alere i detected 63.8% and Roche cobas Liat detected

TABLE 4 CLIA-waived nucleic acid amplification tests for influenza A and B viruses and/or RSV and other respiratory viruses[a]

Kit	Manufacturer	Method	Viruses or species detected (influenza target genes)[b]	Specimens[c]	Instrument[d]	Assay time (min)	Comments
Alere i influenza A & B	Alere, Scarborough, ME, USA	Nicking enzyme isothermal NAAT	Influenza A & B (Flu A: PB2; Flu B: PA)	NS direct	Alere i instrument	15	Foam swabs provided are preferred. Insert swab directly into the sample receiver in the instrument, not into VTM
Alere i RSV	Alere, Scarborough, ME, USA	Nicking enzyme isothermal NAAT	RSV	NPS	Alere i instrument	15	Swabs in kit preferred. Test swab directly or after elution in VTM. Intended for use in patients <18 and ≥60 years of age
Accula Flu A/Flu B	Mesa Biotech, Inc., San Diego, CA	RT-PCR	Influenza A & B (Flu A: PB2; Flu B: M)	NS direct	Accula dock	30	RT-PCR followed by hybridization and colorimetric visualization on a test strip
BioFire FilmArray RP EZ	BioFire Diagnostics, Salt Lake City, UT	Multiplex nested PCR	Adenovirus, CoV, hMPV, RV/EV, influenza A and B, A/H1, A/H3, A/H1-2009, PIV, RSV, plus *Bordetella pertussis*, *Chlamydophila pneumoniae*, *Mycoplasma pneumoniae* (Flu A: M, HA; Flu B: HA)	NPS	FilmArray 2.0 instrument	60	Adenovirus sensitivity improved in v.1.7, but may still be suboptimal
cobas Liat influenza A/B	Roche, Branchburg, NJ	Real-time RT PCR	Influenza A & B (Flu A: M; Flu B: NSP)	NPS	cobas Liat system	20	Amplification curves and estimated cycle threshold displayed
cobas Liat influenza A/B & RSV	Roche, Branchburg, NJ	Real-time RT PCR	Influenza A & B, RSV (Flu A: M; Flu B: NSP)	NPS	cobas Liat system	20	Amplification curves and estimated cycle threshold displayed
Xpert Xpress Flu	Cepheid, Sunnyvale, CA, USA	Real-time RT PCR	Influenza A & B (Flu A: M, PB2, PA; Flu B: M, NSP)	NPS, NA, NW	GeneXpert systems GXII and GXIV	30	Instruments with two (GXII) or four (GXIV) independent modules. No C_T values provided in waived tests
Xpert Xpress Flu/RSV	Cepheid, Sunnyvale, CA, USA	Real-time RT PCR	Influenza A & B, RSV (Flu A: M, PB2, PA; Flu B: M, NSP)	NPS, NA, NW	GXII and GXIV	30	GXII and GXIV. No C_T values provided in waived tests

[a]Information from package inserts and manufacturers' websites.
[b]PB2, RNA polymerase subunit 2; PA, RNA polymerase subunit; M, matrix; HA, hemagglutinin; NSP, nonstructural protein; CoV, coronavirus; hMPV, human metapneumovirus; RV, rhinovirus; EV, enterovirus; PIV, parainfluenza virus; RSV, respiratory syncytial virus.
[c]NS, nasal swab; NPS, nasopharyngeal swab; NA, nasal aspirate; NW, nasal wash.
[d]All instruments except GXII and GXIV accommodate one sample at a time.

97.9% of influenza virus positives (31). In a study by Nolte et al., also using NPS in VTM, Alere i detected 71.4% of influenza A and 93.3% of influenza B positives, whereas Roche cobas Liat detected 100% for both influenza A and B. The reference standard used was BioFire FilmArray RP. The poor detection rate for influenza A virus in this study was attributed to the numerous samples with low viral loads (13). Thus, when low-positive samples were included, Alere i sensitivity was shown to be lower than that for Roche cobas Liat and BioFire FilmArray. Subsequently, Chen et al. compared Alere i to Xpert Xpress Flu and reported sensitivities of 97.4% for influenza A and 81.5% for influenza B viruses for Alere i, and 100% and 96.3%, respectively, for Cepheid Xpert Xpress Flu (32). Half of the samples in this study were from children. Again, all of these studies were performed in laboratories, not at POC, and used nonwaived samples for Alere i, i.e., swabs diluted in VTM rather than direct nasal swabs.

One Alere i RSV study tested pediatric patients in the POC setting (12). The sensitivity overall for RSV was 93% compared to that for rtRT-PCR, and RSV positives were available in 5 min. However, positives with low viral loads (mean C_T, 31.1) were missed (12). A second study, performed on frozen samples by laboratory personnel, reported 100% sensitivity of Alere i RSV in young children compared to that for rtRT-PCR. The results were available within 5 to 7 min for positive samples (C_T value of $<$25) (33).

Roche cobas Liat is, at the time of this writing, the only waived NAAT besides Alere i with at least one published study performed in the POC setting. In a multisite POC study involving 12 locations (8 clinics and 4 EDs), Roche cobas Liat influenza A/B & RSV performed by nonlaboratory staff showed sensitivities of 99.6%, 99.3%, and 96.8% for influenza A, B, and RSV, respectively, and specificities of 97.5%, 99.7%, and 98.8%, respectively. The comparator test was the laboratory-based Prodesse ProFlu+ rtRT-PCR (Hologic, MA, USA) (34). Only 1.8% of the initial results were invalid, indicating robust performance at POC. In the same study, rapid antigen immunoassay testing for influenza A, B, and RSV had relatively high sensitivities of 79.7%, 80.0%, and 87.1%, respectively, indicating excellent sample quality; indeed, half of the samples were from children. Remarkably, the Roche cobas Liat showed greater sensitivity than the reference rtRT-PCR and was superior when testing low-positive samples (C_T values of $>$30), despite being performed by nonlaboratory personnel. In this same study, it was determined that a mutation in the circulating H3N2 strains in the 2014 to 2015 season resulted in false negatives in the Prodesse ProFlu+ reference test, the Roche cobas Liat, Cepheid Xpert Flu, and other NAAT (35). However, more false negatives were seen with Prodesse ProFlu+ than with the cobas Liat.

Cepheid Xpert Xpress Flu is an improved and faster version of the previous Cepheid Xpert Flu test, which had a significant drop in sensitivity in the 2014 to 2015 season as described above. Similar to the Roche cobas Liat, Cepheid has two options: Xpert Xpress Flu and Xpert Xpress Flu/RSV. A recent comparison of Cepheid Xpert Xpress and Roche cobas Liat, conducted in the laboratory and not at POC, showed sensitivities for influenza A, B, and RSV of 100%, 97.8%, and 100%, respectively, for Xpert Xpress, and 100%, 100%, and 100% for Roche cobas Liat (36). Specificities were 99.3 to 100% for both tests. Throughput was much higher for Cepheid Xpert in this report, since a large 16-module Cepheid GeneXpert laboratory instrument was used, compared to a single Roche cobas Liat CLIA-waived POC instrument. There are no studies at this writing of Cepheid Xpert Xpress performed at POC by nonlaboratory staff.

The BioFire FilmArray RP EZ, which detects 14 respiratory viral and bacterial pathogens (see Table 4), is a CLIA-waived version of the FDA-cleared BioFire FilmArray respiratory panel (RP). It is designed to run on a single computer/instrument configuration (EZ configuration) of the BioFire FilmArray 2.0 instrument, which accommodates one sample at a time and takes 60 min to complete. Compared to the nonwaived BioFire FilmArray RP, the waived version does not differentiate either the four parainfluenza virus types or the four coronaviruses. The BioFire FilmArray RP has shown sensitivities and specificities similar to those of real-time PCR, except for adenovirus (37). However, a newer version of the RP, v.1.7, has been modified to detect adenovi-

ruses more efficiently (37, 38). One would expect the sensitivity and specificity of the BioFire RP EZ to be similar to those of the BioFire RP, but reliability in the POC setting performed by nonlaboratory personnel has not yet been published.

The Mesa Biotech Accula Flu A/Flu B is the most recently approved test, and no studies are available for review at this writing. However, the Accula Flu A/Flu B 510(k) substantial equivalence determination decision summary claims comparable performance to the Alere i influenza A & B assay. The Accula test cassette, containing all enzymes and reagents, is inserted into the Accula dock instrument, and then an unprocessed direct nasal sample is added, and the dock lid is closed. The system integrates nucleic acid extraction, amplification, and hybridization in a self-contained and automated system, and the firmware controls fluid flow into the various chambers of the cassette. Results are available in 30 min or less. Similar to Alere i, only direct nasal swabs are approved for POC testing, and the dock instrument accommodates one sample cassette at a time.

Advantages and limitations of NAAT at POC. With results in 15 to 30 min or less for the 7 tests that detect influenza and/or RSV, the assay time for NAAT is similar to the time required for rapid antigen tests. In addition, the waived NAAT tests may be simpler to perform with less hands-on time than some rapid antigen tests. Waived instruments are LIS capable for Roche Liat and Cepheid Xpert Xpress, but not at this writing for Alere i, Accula Mesa Biotech, or FilmArray RP EZ.

From the reported sensitivity studies to date, Cepheid Xpert Xpress and Roche cobas Liat, both rtRT-PCR assays, perform with similar high sensitivities to the best extracted real-time PCR assays performed in the laboratory setting. Since BioFire FilmArray RP EZ uses the same test and instrument as the BioFire FilmArray RP, similar performance is anticipated. Alere i performs well on samples with C_T values of $<$27 to 29, and the testing of direct swabs may enhance performance. The variable sensitivity of Alere i in published studies is likely due to the frequency of inclusion of samples with higher C_T values (low viral load). Mesa Biotech Accula Flu A/Flu B claims substantial equivalence to Alere i performance. The specificities of all NAAT are uniformly high.

While the risk of contamination has been greatly reduced in laboratories with the current closed NAAT systems operated by skilled technologists, problems could arise in POC settings when performed by nonlaboratory personnel. Instruments, counters, and hands can become contaminated from strong-positive samples or controls, potentially by influenza virus in vaccines, or by amplicons if amplified materials are not disposed of properly. Problems with the performance of waived tests in general have been documented in Centers for Medicare & Medicaid Services (CMS) surveys and could impact NAAT as well, such as poor training, frequent staff turnover, the failure to follow manufacturers' instructions, a lack of understanding of good laboratory practices, and the failure to perform quality control (QC) testing or instrument maintenance (39).

While the actual costs depend on negotiated contracts, test volumes, and other factors, the reagent costs are usually significantly higher for molecular kits than for antigen tests, and instruments, with attendant annual service contracts, can be quite expensive (10). Sample throughput is limited, with most instruments accommodating one sample at a time. Thus, to maintain a rapid time to result, multiple instruments may be required during peak periods, but may sit unused outside the influenza season. Invalid results, necessitating repeats, as well as instrument failures, can be problematic with NAAT and consume time and resources.

As with other diagnostic testing for viruses, molecular assays have been shown to be vulnerable to genomic drifts and shifts, leading to performance variation from year to year. In 2014 to 2015, decreased sensitivity was reported with multiple commercial molecular assays, including the Cepheid GeneXpert Flu and Roche cobas Liat, for several clades of the 3C genetic group of influenza A H3N2 compared to that for archived virus from previous years (35). This led to improvements in the tests, including the use of two influenza A virus targets in Cepheid Xpert Xpress. However, the threat of genetic change remains. As noted above for antigen testing, close monitoring of

circulating strains in each influenza season will be key to prompt identification of variations in molecular test performance.

CONCLUSIONS

The era of POC and waived testing for influenza A and B viruses and RSV began with simple immunoassays and now includes digital immunoassays. Despite the lower sensitivity, these tests retain the advantages of simplicity, with either inexpensive readers or no equipment requirements, low reagent and operating costs, and reasonable performance when samples contain high viral loads, such as in young children. RIDTs also have utility in identifying influenza as a cause of respiratory outbreaks, such as in nursing homes, cruise ships, and schools. Driven by the FDA reclassification of RIDTs and competition from POC NAAT, innovative efforts to improve the sensitivity of POC immunoassays for influenza and RSV can be expected to accelerate if manufacturers hope to remain competitive (40).

In recent years, POC NAAT methodologies have emerged and progressed rapidly. The most sensitive molecular testing can now be done on site, in the ED, clinics, and large physician practices by simply adding a sample to a receiver, cartridge, pouch, or cassette, which is then inserted into an instrument, with results in a time frame similar to that of rapid antigen tests. However, the costs of NAAT are substantially greater at present. To justify the increased expenditures, it is essential that test results are linked to actions to guide appropriate therapy, ancillary testing, and infection prevention in order to improve patient care and reduce overall costs for the patient and the community. Studies documenting real, rather than hypothetical, interventions and outcomes should be performed.

Lastly, influenza virus mutations remain a constant concern regardless of the methodology. A partnership between public health, the medical community, and industry will be key to early detection and corrective action, not only for rapid antigen tests but also for molecular assays, through annual reactivity testing of circulating strains. Respiratory virus testing is a rapidly changing area for both the viruses and the technology, and the reader is advised to consult the CDC and FDA websites for the latest guidance.

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