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RESEARCH ARTICLE

Evaluation of high-throughput digital lateral flow immunoassays for the detection of influenza A/B viruses from clinical swab samples

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Abstract

We evaluated the performance of new high-throughput digital lateral flow immunoassays (LFIAs) detecting influenza antigens and compared them with those of the widely used digital LFIA and the rapid nucleic acid amplification test (NAAT). We tested 199 clinical nasopharyngeal (nasal) swab samples using three LFIA tests (BD Veritor Plus, STANDARD F Influenza A/B FIA, and ichroma TRIAS) and the rapid NAAT (ID NOW Influenza A & B2). Agreements and clinical performances (sensitivity and specificity) were evaluated based on the results of reverse transcriptase-polymerase chain reaction (RT-PCR) and verification panel. The agreement of each test with RT-PCR was moderate to almost perfect. The sensitivity of ID NOW was significantly higher than that of LFIAs ($P = .0005$, .0044, and .0026 for influenza A and $P = .0044$, .0026, and .0044 for influenza B, respectively). The specificities were not significantly different between the four tests ($P > .05$). However, the reference panel suggests that ichroma TRIAS test is more sensitive than the other two LFIA tests. All three LFIA assays performed similarly with no false positives against influenza A. For influenza B, ichroma TRIAS had 2 of 166 false positives whereas there were no false positives for the other two LFIA tests. Influenza antigen digital LFIAs have advantages in terms of the workflow when simultaneous tests are required. Rapid NAAT has higher sensitivity, while new antigen LFIAs are efficient and high-throughput. It is recommended that users select appropriate methods and algorithms according to the number of specimens and laboratory conditions in each clinical laboratory.

KEYWORDS

influenza virus, lateral flow immunoassay, nucleic acid amplification tests, rapid influenza diagnostic test

1 | INTRODUCTION

Despite the increased coverage of influenza vaccination, we experience influenza epidemics every year as the virus continuously evolves to evade pre-existing immunity at the population level.[1] Since the respiratory symptoms of flu are nonspecific and variable, clinical manifestations alone are not enough to differentiate the influenza virus from other respiratory pathogens.[2] Numerous methods can be

Abbreviations: LFIA, lateral flow immunoassay; NAAT, nucleic acid amplification test; RT-PCR, reverse transcriptase-polymerase chain reaction; RIDT, rapid influenza diagnostic test; TAT, turnaround time; POCT, point-of-care testing.

used to identify influenza virus in respiratory secretions, including cell culture, immunoassays for detecting antigen, and nucleic acid amplification tests (NAATs).[3] Currently, several types of rapid influenza diagnostic tests (RIDTs) for quick and accurate identification are commercially available. RIDTs are advantageous as rapid diagnostic tests not only because they help with prompt initiation of antiviral therapy, but also because they prevent the need for additional diagnostic tests, unnecessary antibiotic use, longer hospitalization, and infection transmission.[4-7]

Lateral flow immunoassay (LFIA) for detecting influenza antigen is a rapid and simple test, but has limitations such as low sensitivity and subjective interpretation that is often qualitative and not quantitative.[8] The development of automated LFIAs with digital scanning has enabled objective readings but still presents with limitations concerning sensitivity as well as delays, especially when several specimens are being processed during epidemics. Recently, the combination of fluorescence labeling and a portable reading system in LFIA has improved the sensitivity and speed of the assay compared with traditional immunochromatic methods.[9-13] While NAAT-based assays are considered as the gold-standard confirmation method that can replace cumbersome viral culture, they are time-consuming and require skilled personnel. Novel rapid NAAT using modified reverse transcriptase-polymerase chain reaction (RT-PCR) or isothermal amplification techniques can shorten the turnaround time (TAT) of NAAT, and include molecular assays as one of the confirmative measures during point-of-care testing (POCT).[3,14-16] Nonetheless, LFIAs are portable, stable, cost-effective, and easy to use. The simplicity of commercially available LFIA-based tests for the initial assessment of influenza-positive samples is ideal in clinics that do not have immediate access to the clinical instruments and NAAT technologies. Influenza-specific LFIAs are even being multiplexed to detect different strains of flu on the same strip, and this technology is needed to gauge emerging pandemics.[17,18] Knowing the performance of various LFIA technologies in clinical and in-field settings will help policymakers and private industries to improve the LFIA development process and even the reader technology.

Although RIDT was originally developed as a tool for POCT, physicians in many institutions still send patients' respiratory specimens to central laboratories expecting rapid reports. Thus, the capabilities for simultaneous handling, incubation, and measurement are also important considerations in RIDT devices. Recently, some high-throughput digital RIDTs using the LFIA technique have also been introduced.[19] There have been many evaluation reports on RIDTs dealing with the LFIA and rapid NAATs, but there have been no reports on the newly developed high-throughput digital LFIAs for detecting influenza antigens. Thus, we evaluated the performance of the new high-throughput digital LFIAs (STANDARD F Influenza A/B FIA [SD Biosensor, Suwon, Korea] and ichroma TRIAS ([Boditech Med, Chuncheon, Korea]) for detecting influenza antigens, and compared them with those of the widely used digital LFIA (BD Veritor Plus; Becton, Dickinson and Company, Sparks, MD) and the rapid NAAT ID NOW Influenza A & B2 (Abbott Molecular, Des Plaines, IL).

2 | MATERIALS AND METHODS

2.1 | Clinical samples

This study was approved by the Institutional Review Board (2019-04-016) of the Konkuk University Medical Center, Seoul, Korea. We included 199 nasopharyngeal or nasal swab samples in universal transport media that were submitted to our laboratory for influenza testing from patients that presented respiratory symptoms from January to March 2019. As a routine diagnostic procedure, each sample was tested using BD Veritor Plus, and these samples were additionally tested using three rapid tests, STANDARD F Influenza A/B FIA, ichroma TRIAS, and ID NOW Influenza A & B2. All samples were confirmed by RT-PCR to be influenza-positive or -negative. STANDARD F and ichroma TRIAS were simultaneously tested when BD Veritor Plus was performed. ID NOW and RT-PCR were performed using samples that were stored at −70°C. The data were analyzed anonymously without patient information, and this study did not require study-specific intervention or any other type of intervention. Therefore, written informed consent from enrolled patients was exempted.

2.2 | Influenza rapid test verification panel

We used the influenza rapid test verification panel I (ZeptoMetrix Corporation, Franklin, MA) as a control to comparatively assess the reactivity of each test to each strain at various concentrations. This panel was prepared from inactivated viral culture fluids and is composed of 20 members representing various strains (8 negative, 7 influenza A; Brisbane/59/2007 [H1N1], Brisbane/10/2007 [H3N2], Perth/16/2009 [H3N2], Solomon Islands/03/2006 [H1N1], New Caledonia/20/1999 [H1N1], Swine NY/01/2009 [H1N1], Swine Canada/6294/2009 [H1N1], 5 influenza B; Lee/40, Florida/02/2006, Brisbane/33/2008, and 2 samples of Panama/45/1990). Each panel was diluted with phosphate-buffered saline (1:10, 1:50, 1:100, 1:500, and 1:1000) and tested with each assay.

2.3 | Assays

The BD Veritor Plus System (Veritor Plus) is a digital LFIA using colloidal metal particles for the direct and qualitative detection of influenza A and B viral nucleoprotein antigens. Influenza A or B viral antigens bind to anti-influenza Ab conjugated to detector particles in the A+B test strip, and the antigen-conjugate complex migrates across the test strip to the reaction area and is captured by an antibody on the membrane. The assay-specific instrument analyzes and corrects for nonspecific binding, and detects positives not recognized by the naked eye to provide an objective digital result.

STANDARD F Influenza A/B FIA (STANDARD F) is an LFIA using fluorescence signal detection (europium) to detect influenza virus nucleoproteins. This assay has two test lines (A and B) and a control line that is coated with monoclonal anti-influenza A, monoclonal anti-influenza B, and polyclonal mouse immunoglobulin G each. The influenza A/B viral antigen in the patient sample will react with the

europium-conjugated monoclonal anti-influenza A/monoclonal anti-influenza B in the conjugation pad and form antibody-antigen fluorescence particle complexes. These complexes move along to the membrane to be captured by the anti-influenza A/anti-influenza B on the test line and emit fluorescence. The intensity of the fluorescence emitted onto the membrane is scanned by the STANDARD F Analyzer (SD Biosensor). Either both the reaction and scanning (standard test mode) or only scanning (read-only mode) can be performed using the analyzer.

The ichroma TRIAS influenza A+B (ichroma TRIAS) is also a LFIA using fluorescence labeling with lanthanide (europium) chelates. The cartridge contains a test strip, which contains the membrane with anti-human influenza A/B, while the control line contains chicken immunoglobulin Y (IgY). The test strip contains anti-influenza A/B fluorescence conjugate and anti-chicken IgY fluorescence conjugate. The cartridge can be inserted into the ichroma II instrument (Boditech Med) for signal scanning before or after 10 minutes of incubation, depending on the number of simultaneous tests (single- or multi-mode).

The ID NOW Influenza A & B2 rapid molecular in vitro diagnostic test (ID NOW) utilizes an isothermal NAAT for the qualitative detection and discrimination of influenza A and B viral RNA in samples. This assay is composed of a sample receiver with elution buffer; a test base made up of two sealed reaction tubes containing reagents required for the amplification of influenza A (PB2 segment), influenza B (PA segment), and internal controls; a transfer cartridge for transfer of the eluted sample to the test base; and the instrument. Fluorescent-labeled molecular beacons were used to specifically identify each of the amplified RNA targets.

For RT-PCR, RNA was extracted using QIAamp MinElute Virus Spin (QIAGEN, Hilden, Germany) and RT-PCR was performed using Biosewoom Real-Q Flu (BioSewoom, Seoul, Korea) in which samples above the 38-cycle threshold are considered positive according to the manufacturer's instruction. ID NOW-positive and RT-PCR-negative samples were verified by additional RT-PCR testing using Anyplex II RV16 RT-PCR (Seegene, Seoul, Korea). Samples that appeared positive in any RT-PCR assay were considered positive, and samples that appeared negative in both RT-PCR assays were considered negative. The sensitivity and specificity of each assay were calculated as test positive/positive samples and test negative/negative samples, respectively. All assays in this study are CE-approved and clinically available.

2.4 | Statistical analysis

Agreement between assays were determined using Cohen's kappa (agreement: <.20, none; 0.21-0.39, minimal; 0.40-0.59, weak; 0.60-0.79, moderate; 0.80-0.90, strong; >0.90, almost perfect).[20] The sensitivity and specificity with 95% confidence interval for each assay were determined based on the results of RT-PCR. McNemar's test was used to analyze the statistical differences in the sensitivity and specificity between assays. Statistical analysis was performed using MedCalc Statistical Software (version 12.3.0, MedCalc Software, Mariakerke, Belgium). *P* values less than .05 were considered statistically significant.

3 | RESULTS

3.1 | Agreement and performance of each test based on RT-PCR

For the detection of influenza A, the agreement of each test with RT-PCR was moderate to almost perfect (kappa .774, .844, .827, and .986 for Veritor Plus, STANDARD F, ichroma TRIAS, and ID NOW, respectively). The sensitivities of the three LFIAs (68.9%, 77.8%, and 75.6% for Veritor Plus, STANDARD F, and ichroma TRIAS, respectively) were not significantly different ($P > .05$). The sensitivity of ID NOW was 100.0% and significantly higher than that of the LFIAs ($P = .0005$, .0044, and .0026, respectively). Out of 14 samples that were positive for influenza A by RT-PCR but were negative by the LFIAs, 10 were negative in all LFIAs. The specificities were not significantly different between tests ($P > .05$; Table 1). All three LFIAs showed no false positives against influenza A.

TABLE 1 Agreement and performance of each assay for influenza A based on RT-PCR

	RT-PCR				
Assays	Positive (n = 45)	Negative (n = 154)	Kappa	Sensitivity	Specificity
BD veritor Plus					
Positive	31	0	.774 (0.663-0.885)	68.9% (53.4%-81.8%)	100.0% (97.6%-100.0%)
Negative	14	154			
STANDARD F					
Positive	35	0	.844 (0.751-0.937)	77.8% (62.9%-88.0%)	100.0% (97.6%-100.0%)
Negative	10	154			
Ichroma TRIAS					
Positive	34	0	.827 (0.729-0.915)	75.6% (60.5%-87.1%)	100.0% (97.6%-100.0%)
Negative	11	154			
ID NOW					
Positive	45	1	.986 (0.958-1.000)	100.0%* (92.1%-100.0%)	99.3% (96.4%-99.9%)
Negative	0	153			

Note: Each value is presented with its 95% confidence interval.
Abbreviation: RT-PCR, reverse transcriptase-polymerase chain reaction.
*$P = .0005$, .0044, and .0026 compared with BD veritor Plus, STANDARD F, and Ichroma TRIAS by McNemar's test.

TABLE 2 Agreement and performance of each assay for Influenza B based on RT-PCR

	RT-PCR				
Assays	**Positive (n = 33)**	**Negative (n = 166)**	**Kappa**	**Sensitivity**	**Specificity**
BD veritor Plus					
Positive	21	0	0.745 (0.610-0.880)	63.6 (45.2%-79.6%)	100.0% (97.8%-100.0%)
Negative	12	166			
STANDARD F					
Positive	20	0	0.720 (0.578-0.861)	60.6% (42.1%-77.1%)	100.0% (97.8%-100.0%)
Negative	13	166			
Ichroma TRIAS					
Positive	21	2	0.710 (0.569-0.862)	63.6% (45.1%-79.6%)	98.8% (95.6%-99.9%)
Negative	12	164			
ID NOW					
Positive	31	6	0.861 (0.768-0.955)	93.9%* (79.8%-99.3%)	96.4% (92.3%-98.7%)
Negative	2	160			

Note: Each value is presented with its 95% confidence interval.
Abbreviation: RT-PCR, reverse transcriptase-polymerase chain reaction.
*P = .0044, .0026, and .0044 compared with BD veritor Plus, STANDARD F and Ichroma TRIAS by McNemar's test.

For the detection of influenza B, the agreement of each test with RT-PCR was moderate to strong (kappa 0.745, 0.720, 0.710, and 0.861 for Veritor Plus, STANDARD F, ichroma TRIAS, and ID NOW, respectively). The sensitivities of the three LFIAs (63.6%, 60.6%, and 63.6% for Veritor Plus, STANDARD F, and ichroma TRIAS, respectively) were not significantly different ($P > .05$). The sensitivity of ID NOW was 93.9% and significantly higher than that of the LFIAs ($P = .0044$, .0026, and .0044, respectively). For influenza B, 12 were negative in all LFIAs out of 13 samples that were positive for influenza A by RT-PCR but were negative by the LFIAs. The specificities were not significantly different ($P > .05$; Table 2), but ichroma TRIAS had 1.2% (2 out of 166) false-positive reactions for influenza B compared to RT-PCR.

3.2 | The sensitivity of each assay to the verification panel including various strains and negative samples

All assays showed positive results for the seven influenza A and five influenza B strains included in the panel. The final dilutions that showed positive results were variable according to strains and assays. LFIAs targeting influenza antigens (Veritor Plus, STANDARD F, and ichroma TRIAS) showed positive results at 1:50 to 1:100 dilution, 1:10 to 1:500 dilution, and 1:100 to 1:500 dilution, respectively, for all influenza A/B antigens included in the panel. The ichroma TRIAS was more sensitive in detecting some of the strains in the panel (Brisbane/10/2007, Solomon Islands/03/2006, Swine NY/01/2009, Swine Canada/6294/2009, Lee/40, and Panama/45/1990) at a greater dilution than the other two LFIA tests. The rapid NAAT (ID NOW) showed positive results from 1:100 to 1:1000 dilutions. For negative samples in the panel, all assays showed negative results (Table 3).

3.3 | Comparison of assays on workflow

The specifications of each assay are presented in Table 4. In addition to the underlying principle, which can influence the assay performance, these assays also vary based on their reaction site (outside or only in the instruments) and the number of simultaneous tests that can be performed. We compared the time for performing single and multiple tests simultaneously. The hands-on time before the reaction was similar across the assays. The reaction and reading times for single tests were also similar (10 minutes) but were different for simultaneous tests. The possibility to perform the reaction outside can reduce the time required to use the instrument and improve the efficiency. Assays that can handle a high number of simultaneous tests such as STANDARD F, which does not require the strip to be replaced, can improve the efficiency of the workflow, especially when the number of tests is increased.

4 | DISCUSSION

In this study, we compared the clinical performances of rapid tests for influenza viruses, including a colorimetric digital LFIA (Veritor Plus), digital LFIAs with fluorescence labeling (STANDARD F and ichroma TRIAS), and isothermal NAAT (ID NOW). LFIA using fluorescence labeling is an emerging method and is now widely used in clinical diagnosis. Newly reported lanthanide chelates that can be used as fluorescence labels for LFIA with a narrow emission spectrum, a broad excitation spectrum (613 and 333 nm), and a large Stokes shift allow easy discrimination and eliminate the background fluorescence associated with the use of many existing fluorophores.[21,22] The longer half-life of europium nanoparticles enables a wider time-resolved fluorescence reading system and leads to a wider detection range, as well as higher sensitivity and accuracy.[23-25] In this study, antigen tests using fluorescence labels (STANDARD F and

TABLE 3 Reactivity of each assay for a panel including various strains and negative samples

	Strain	BD veritor Plus	STANDARD F	Ichroma TRIAS	ID NOW
A	Brisbane/59/2007	1:100	1:500	1:500	1:1000
	Brisbane/10/2007	1:100	1:100	1:500	1:500
	Perth/16/2009	1:100	1:100	1:100	1:1000
	Solomon Islands/03/2006	1:50	1:50	1:100	1:100
	New Caledonia/20/1999	1:100	1:100	1:100	1:1000
	Swine NY/01/2009	1:100	1:100	1:500	1:500
	Swine Canada/6294/2009	1:100	1:100	1:500	1:1000
B	Lee/40	1:50	1:50	1:100	1:1000
	Florida/02/2006	1:100	1:100	1:100	1:1000
	Brisbane/33/2008	1:100	1:100	1:100	1:1000
	Panama/45/1990	1:50	1:10	1:100	1:1000
	Panama/45/1990	1:50	1:50	1:100	1:1000
Negative (n = 8)		All negative	All negative	All negative	All negative

Note: The viral culture fluid from positive strains were diluted to 1:10, 1:50, 1:100, 1:500, 1:1000, and final dilution of positive results were indicated.

ichroma TRIAS) showed higher sensitivity for clinical specimens compared with BD Veritor Plus for influenza A, but it was not statistically significant (Tables 1 and 2). In the experiment with the sensitivity panels, ichroma TRIAS showed positive reactions at higher dilutions in many strains compared with other antigen tests, suggesting higher sensitivity (Table 3). Rapid NAAT showed significantly higher sensitivity in clinical specimens for both influenza A and B (Tables 1 and 2). Most of the samples that were only positive in RT-PCR showed high C_t in RT-PCR (>30). Moreover, half of these samples (6 of 12 in influenza B) were only positive in one RT-PCR kit, suggesting a very low virus load on the requested samples. The higher sensitivity of NAAT was also observed in sensitivity panel testing, presenting one to twofold higher than antigen assays (1:1000 in nine strains, 1:500 in two strains, and 1:100 in one strain; Table 3). This finding is consistent with our current knowledge that molecular methods generally have a higher sensitivity than immunoassays. Even though false positives (6 out of 166 negative samples in both RT-PCR kits) were detected using ID NOW in the clinical specimens, we also could not rule out the false negatives by RT-PCR for samples with very low concentrations of influenza viruses.

These rapid tests have different advantages and disadvantages, and these are predicted from their different specifications as described in Table 4. Appropriate tests may be selected in each laboratory depending on the number of tests requested in each epidemic, the number of technicians assigned to the test, the TAT expected by the clinician, and the number of patients in each institution. During an influenza epidemic, some laboratories handle more than a hundred specimens a day with a small number of technicians on duty.[26] For example, during the pandemic influenza A (H1N1) in the 2009-2010 season, inpatient visits increased

TABLE 4 Comparison of the specification of each assay

	BD veritor Plus	STANDARD F (F2400)	Ichroma TRIAS	ID NOW
Principle	LFIA	LFIA (Europium)	LFIA (Europium)	Isothermal NAAT
Target	Nucleoprotein	Nucleoprotein	Nucleoprotein	Viral RNA (PB2 and PA segment)
Hand-on time per test	<5 min	<5 min	<5 min	<5 min
Reaction site	Outside/in the instrument	In the instrument	Outside/in the instrument	In the instrument
Reaction time	10 min	10 min (including reading)	10 min	10 min (including reading)
Reading	instrument	instrument	instrument	instrument
Reading by the naked eye	Yes	No	No	No
Reading time	3 s (read-only)	–	15 s (read-only)	–
Simultaneous tests per instrument	1	24 (F2400)	1, 3	1
Time for one test[a]	~10 min	~10 min	~10 min	~10 min
Time for simultaneous 10 tests[a]	<15 min	<15 min	<15 min	100 min
Manual replacing of strip	Needed	Not needed	Needed	Needed
Instrument size (W × L × H), mm	90 × 143 × 76	510 × 566 × 297	116 × 210 × 80	207 × 194 × 145
Cost	Moderate	Moderate	Moderate	Moderate to high

Abbreviations: LFIA, lateral flow immunoassay; NAAT, nucleic acid amplification test.
[a]Reaction and reading time for simultaneous tests after sample preparation. Time can be reduced if multiple instruments are applied.

15 to 20 times, outpatient visits increased 10-fold, and mean diagnostic costs increased 58.5 times compared with that in the two previous seasons in Korea.[27] This means that there are situations in which multiple specimens are processed simultaneously. In this case, it can be more efficient to have several strips react at the same time outside of the instrument and only facilitate measurement inside the instrument. This is possible for Veritor Plus, ichroma TRIAS, and the F200 model of the STANDARD F series. Among the STANDARD F series, the F2400 equipment, which is a high-throughput test, can process 24 strips at once and allow rapid reactions and measurements to be facilitated simultaneously. The TAT can be further shortened in the case of strong positive reactions because a positive result can be obtained within 1.5 to 5 minutes. The color reaction on the Veritor Plus strip can be measured via the naked eye to shorten the TAT for definitive positive reactions. This assay also has an advantage in that the instrument required is very small in size.

Generally, it is common to perform an antigen test first before performing NAATs considering the high price and TAT of NAATs. However, ID NOW, the rapid NAAT targeting influenza RNA, showed similar aspects in terms of simplicity and speed when compared with the antigen rapid tests, while showing significantly higher sensitivity.[15,16] Considering the frequency of false negatives in rapid antigen tests, it is also effective to perform NAATs primarily when the patient is in need of prompt confirmation and subsequent antiviral treatment.[6,7,28] However, given the fact that rapid NAAT instruments can process only one sample at a time until the test of one sample is finished, it is also appropriate to conduct high-throughput tests that can process multiple specimens simultaneously, especially during influenza epidemics.[29]

In summary, both antigen LFIAs and rapid NAATs showed satisfactory performances. Rapid NAAT has higher sensitivity, while antigen LFIAs are efficient and high-throughput. It is advisable to select appropriate methods and algorithms according to the number of specimens and the conditions in each clinical laboratory.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

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