Video Article

Rapid Molecular Detection and Differentiation of Influenza Viruses A and B

Caitlin C. Otto[1], Samuel E. Kaplan[1], Jeffrey Stiles[1], Albina Mikhlina[1], Cindy Lee[1], N. Esther Babady[1], Yi-Wei Tang[1]

[1]Department of Laboratory Medicine, Memorial Sloan Kettering Cancer Center

Correspondence to: Yi-Wei Tang at tangy@mskcc.org

Abstract

Influenza is a contagious respiratory illness caused by influenza viruses A and B in humans and causes a significant amount of morbidity and mortality every year. The Influenza A and B assay was the first CLIA-waived molecular rapid flu test available. The Influenza A and B test works by employing isothermal amplification with influenza-specific primers followed by target detection with molecular beacon probes. Here, the performance of the Influenza A and B assay on frozen, archived nasopharyngeal swab (NPS) specimens stored in viral transport medium (VTM) were compared to a respiratory panel assay.

The performance of the Influenza A and B assay was evaluated by comparing the results to the respiratory panel reference method. The sensitivity for total influenza virus A was 67.5% (95% CI (CI), 56.6-78.5) and the specificity was 86.9% (CI, 71.0-100). For influenza virus B testing, the sensitivity and specificity were 90.2% (CI, 68.5-100) and 98.8% (CI, 68.5-100), respectively.

This system has the advantage of a significantly shorter test time than any other currently available molecular assay and the simple, pipette-free procedure runs on a fully integrated, closed, small-footprint system. Overall, the Influenza A and B assay evaluated in this study has the potential to serve as a point-of-care rapid influenza diagnostic test.

Video Link

The video component of this article can be found at https://www.jove.com/video/54312/

Introduction

Influenza virus infections result in a significant amount of morbidity and mortality each year[1,2,3]. Uncomplicated influenza is characterized by constitutional and respiratory symptoms such as fever, myalgia, headache, and non-productive cough[4,5]. Older individuals, young children, immunocompromised patients, and patients with underlying comorbidities are at a higher risk for serious complications such as pneumonia, myocarditis, central nervous system disease, or death[6,7].

Influenza infection is unique from other respiratory viruses in that prompt administration of antiviral therapy within 48 h of symptom onset can reduce the disease severity and length[8]. Rapid identification of influenza has also been shown to reduce the use of unnecessary antibiotics[9,10]. Further, hospitalized patients with influenza infections must be placed in isolated rooms with appropriate infection control precautions. However, respiratory illnesses caused by non-influenza viruses can be difficult to clinically distinguish from influenza. For this reason, rapid and accurate diagnostic testing for influenza is very important for clinical patient management.

Several assays are available for the detection and identification of influenza viruses. Rapid influenza antigen detection tests (RIDTs) are widely used in clinical practice as point-of-care tests because they are simple to use and provide results within 15 to 30 min[11,12]; however, their sensitivities vary widely (10-80%) depending on the manufacturer and the population being tested, and the influenza type and subtype[13,14,15]. Direct fluorescence assays (DFAs) provide superior sensitivities over RIDTs, but the processing time is greater (~3 hr) and must be completed by skilled technologists[16,17]. Viral culture has been the gold standard for influenza diagnostics and has improved sensitivity over both RIDTs and DFAs[18]. However, influenza viral culture can take anywhere from 2-14 d to complete, diminishing its utility in aiding patient management[19]. Lastly, nucleic acid amplification tests (NAAT) have replaced culture techniques as the new gold standard in influenza diagnostics. NAAT are considered to have the greatest sensitivity for detecting influenza in a few hours. However, NAAT are the most expensive assays and require specialized equipment and technologists to perform[5,20,21,22,23,24,25].

The influenza A and B assay described here is the first CLIA-waived molecular rapid flu test that is readily available. This assay works by employing a Nicking Endonuclease Amplification Reaction (NEAR) that uses isothermal amplification with influenza-specific primers followed by target detection with molecular beacon probes. This assay differentiates influenza A from B, requires 2 min to set up and process one sample, and requires a total of 15 min to complete.

Here, we present the protocol for the Influenza A & B assay. In addition, we provide a sample data set comparing the performance of the Influenza A and B assay on archived nasopharyngeal swab (NPS) specimens stored in viral transport medium (VTM) to another respiratory pathogen panel assay.

Protocol

ETHICS STATEMENT: The use of left-over clinical specimens is approved and follows the guidelines of the Memorial Sloan Kettering Cancer Center Institutional Review Board.

1. Before Running the Assay

NOTE: The Influenza A and B assay is approved for nasopharyngeal swab specimens and for nasopharyngeal swabs stored in viral transport media. Swabs are included in the kit and should be used for optimal performance. However, rayon, foam, flocked swabs or polyester nasal swabs can also be used to collect nasal swab samples.

1. To collect a nasal swab sample, insert the swab into the nostril that has the most visible drainage or that is most congested. With gentle rotation, push the swab into the nostril until resistance is met and rotate several times against the nasal wall before slowly removing from the nostril. Store the swabs and transport them in vials containing 3 ml of viral transport media (VTM).
    NOTE: Careful attention to appropriate specimen collection must be taken as inadequate specimen collection may yield erroneous results.
    NOTE: Swab specimens should be tested as soon as possible after collection. However, they may be stored at room temperature for up to 2 hr or refrigerated at 2-8 °C for up to 24 hr if testing is not immediately available. Freshly collected specimens are ideal for optimal test performance. Improper sample handling, storage, and/or transport may yield erroneous results.
    NOTE: As a precautionary measure, individuals performing this test should treat all specimens as potentially infectious by following universal precautions when handling specimens.
2. Bring all samples to room temperature prior to testing.
    NOTE: All test components are for a single use only and should not be used to perform multiple tests. Do not mix kit components from different lot numbers. Do not use kit reagents past their expiration date.
3. Bring the blue sample receiver to room temperature prior to testing. The orange test base can be tested without the need to warm to room temperature.
4. Leave all test pieces in the foil wrapping until immediately before use. Turn on the instrument by pressing the power button on the side of the instrument. Enter the user-ID and press 'OK.'

2. Running the Test

1. To begin the test process, touch 'Run Test' on the instrument screen. Enter the patient ID by using on-screen keyboard or barcode scanner and press 'OK.' Open the lid and insert the Orange test base into the Orange test base holder.
2. Confirm that the correct test is displayed on the screen and press 'OK.' Insert the blue sample receiver into the blue sample receiver holder.
    NOTE: Do not open the sample receiver before placing in the instrument as this will prevent the elution buffer from reaching the correct operating temperature and may impact test performance.
3. Wait for the sample receiver to warm up. When prompted by the instrument, remove the foil seal and place the patient swab to be tested into the sample receiver.
    NOTE: When removing the foil, place two fingers on the edge of the Sample Receiver to ensure that it stays in place.
4. Vigorously mix the swab in the liquid for 10 sec, pressing the swab head against the side the sample receiver as you mix it to help dislodge the specimen from the swab. Press 'OK' to proceed. If testing swabs stored in viral transport medium, vortex the VTM for 10 sec then add 200 µl to the sample receiver.
5. Press the white transfer cartridge into the blue sample receiver. Continue pressing on the sample receiver until the orange indicator rises.
6. Lift and then connect the transfer cartridge to the test base. Observe the orange indicator descend once the cartridge transfer is attached correctly. Close the Lid and do not open until the "Test Complete" message appears on the screen.

3. Quality Control

1. Touch 'Run QC Test' on the home screen. Select the QC Test to be Run. Confirm the test type to match the QC sample intended for testing by touching 'OK' and following the on screen prompts to complete testing.

4. Result Interpretation

1. After the test run is complete, observe the results of the test displayed on the screen with interpretations for the presence of Influenza A, B, unknown subtypes. For invalid results repeat the test.

5. Maintenance and Cleaning

1. Clean the instrument and the surrounding bench area daily with 70% ethanol or 10% bleach solution. Spray the cleaning solution on a damp, lint-free cloth to clean. Do not pour the solutions directly on the instrument.

Representative Results

In this study, archived NPS specimens were collected from inpatients presenting with influenza-like symptoms at Memorial Sloan-Kettering Cancer Center (MSKCC) during an influenza outbreak between December 15, 2012 and March 1, 2013. The NPS specimens were submitted in 3 ml of VTM and tested as part of routine clinical practice with a molecular assay that detects a panel of respiratory viruses (RP), including Influenza A, A-1, A-3, and B. During the study period, 3,675 NPS specimens were submitted to the MSKCC clinical laboratory for testing. Of those specimens, 45, 425, 37, and 77 tested positive for influenza viruses A-1, A-3, A-un-subtypable (A-u), and B, respectively with the RP. A total of 360 positive specimens, 40 samples from each of the influenza subtypes, and 37 A-u. In addition, 1-2 negative samples that arrived to the laboratory immediately before or after each of the positive specimens were chosen for subsequent testing with the influenza A and B assay.

In total, the Influenza A and B assay detected 79 influenza virus A, 37 influenza virus B, 240 negative, and 4 invalid results (failure of internal controls) (**Table 1**). There were 46 discrepant results with 44 samples positive on RP only and two positive on the Influenza A and B assay only. A third molecular-based influenza assay was performed on the 44 discordant specimens which detected 33/43 (8 influenza virus A, 21 A-u, and 2 influenza virus B) that were positive by the RP and 0/1 positive (influenza virus A) by the Influenza A and B assay. An agreement between two or more assays was considered an accurate identification.

The overall Influenza A and B assay sensitivity for total influenza virus A was 67.5% (95% CI (CI), 56.6-78.5). The sensitivity for the influenza A subtypes was A-1, 84.6% (CI, 58.5-100), A-3, 90.2% (CI, 62.8-100), and A-u, 21.9% (CI, 19.6-24.1). The overall specificity for the influenza virus A was 86.9% (CI, 71.0-100). For influenza virus B testing, the sensitivity and specificity were 90.2% (CI, 68.5-100) and 98.8% (CI, 68.5-100), respectively.

Overall, the mean CT values in specimens with discordant results were significantly higher than those obtained for concordant results (16.4 ± 0.48 vs. 25.7 ± 0.51; p >0.000) (**Figure 1**).
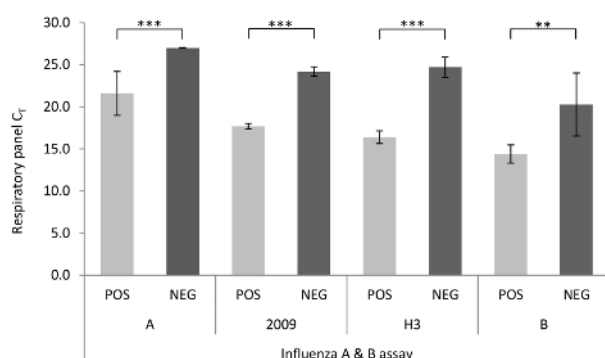

Figure 1: Average CT values determined by RP assay for samples that were positive and negative, respectively by the assay for each influenza subtype. Error bars represent standard deviation. **, p <0.01; ***, p <0.001. Please click here to view a larger version of this figure.

Virus	Genotype	No. detected (Influenza A and B/Reference)[o]				Sensitivity, % (95% CI)	Specificity, % (95% CI)
		+/+	+/-	-/+	-/-		
Influenza A[b]	Total	77	0	35	248	67.5 (54.3-80.8)	86.9 (58.5-100)
	H1N1-2009	33		6		84.6 (56.2-100)	98.2 (19.6-100)
	H3N2	37		4		90.2 (61.3-100)	98.8 (2.00-100)
	Untypable	7		25		21.9 (17.1-26.7)	92.9 (56.6-100)
Influenza B		37	0	4	318	90.2 (61.3-100)	98.8 (2.00-100)

[o] Four invalid Influenza A and B assay results were excluded from the statistical analysis, leaving a total of 356. +, positive; -, negative.

[b] One respiratory pathogen panel-negative sample was identified as influenza virus A by the Influenza A and B assay and confirmed by the reference method.

Table 1: Performance of the Influenza A&B on VTM specimens compared to reference results.

Discussion

Influenza viruses are significant world-wide causes of morbidity and mortality. Rapid and accurate diagnosis of influenza is one of the major keys to managing flu outbreaks during respiratory season. Other antigen-based tests are rapid and easy to perform; however, they have low sensitivities[13]. On the other hand, traditional molecular tests have improved sensitivity, but require more experienced laboratory technologists to perform and are more costly. The Influenza A and B assay described in this study and protocol is a CLIA-waived, rapid molecular test for Influenza A and B. The technology is based on an isothermal-amplification for the detection and differentiation of influenza viruses A and B. We

show representative results to demonstrate the performance of the influenza A and B assay on a panel of archived, frozen NPS VTM samples was evaluated as compared to another molecular-based respiratory panel assay.

The samples that were determined to be "equivocal" or "untypeable" by the RP test had the lowest sensitivity by the Influenza A and B assay. In addition, discordant results were observed more frequently for specimens with higher C_T values in RP assays, suggesting that the lower detection rate by the assay was associated with the lower viral titers. The assay had an improved performance for the detection on influenza virus B over influenza virus A detection with a 90.2% sensitivity and 98.1% specificity. The results here are consistent with previously published literature[26,27,28].

This study has two important limitations. First, samples were stored in VTM for testing on the platform. However, at the time the samples were tested, the platform did not have FDA approval for this sample type. Therefore, the dilution into VTM, may have reduced the detection of low-titer VTM samples. Second, the specimens that were tested with the Influenza A and B assay had been stored at -70 °C for 6 to 9 months prior to testing whereas the RP testing was done on fresh specimens immediately after they were received. It is possible that freezing the specimens further reduced influenza detection in the samples.

The influenza A & B assay has few steps and is simple to perform. However, there are two key steps that must be completed appropriately and that users can troubleshoot if they experience errors. First, when initially setting up the assay, the transfer cartridge must be connected to the test base until they click into place. If these two pieces are not secured appropriately, the run will fail and will need to be repeated from the beginning. Second, the instrument needs to warm up for 2 min prior to adding the sample. After this warm up time, users have 30 sec to add the sample, connect the transfer cartridge to the test base, and close the lid. If the time passes before this step is complete, the instrument will fail the run and the sample set up will have to be repeated from the beginning.

The limitations of the assay include slightly increased hands-on time and lower sensitivity compared to the respiratory panel and does not subtype influenza A. Further, the only specimen types that can be used with the assay are nasopharyngeal swabs or the NPS that are stored in viral transport media; the performance of the assay for any other specimen type, such as lower respiratory specimens, has not been evaluated. It is important to note that all of the steps in this protocol are critical. Any modifications or deviation from the protocol as written may result in lower performance or of the assay and must be assessed by the user. The benefit of the technology is that the instrument has a small footprint, only takes 15 min to perform and, even though the technology is based on molecular methods, does not require special training or qualifications to perform. The Influenza A and B assay evaluated in this study has the potential to serve as an alternative to RIDTs for the diagnosis of influenza. The system has the advantage of a short total test time, has a small footprint and is easy to use. Overall, the design of the instrument is suitable for point-of-care testing.

Disclosures

The authors have nothing to disclose.

Acknowledgements

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