มหาวิทยาลัยรังสิต
เมืองเอก ก.พหลโยธิน
จ.ปทุมธานี 12000

Rangsit University
Muang-Ake, Paholyothin Rd.
Pathumthani 12000, Thailand

T. (66) 2997 2200-30
F. (66) 2533 9470
E. info@rsu.ac.th

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Dear Rujikorn kanlayanadonkit,

I am pleased to inform that paper entitled

"Detection of Influenza A Virus and Influenza B Virus Using Isothermal Reverse Transcriptase-Helicase Dependent Amplification Solana Influenza A+B Assay"

of the authors named
Rujikorn kanlayanadonkit, Acharawan Thongmee, Ekawat Prasomsub, Patamaporn Sukplang, Wanida Pongstaporn and Wasun Chantratita

has been accepted for publication in Interprofessional Journal of Health Sciences (Interprof. J. Health Sci.) formerly Bulletin of Health, Science and Technology (BHST).

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Please note that the accepted paper will be sent to the corresponding author for corrections prior to publishing in the journal.

Thank you for submitting your work to our journal.

Yours sincerely,

Wipaporn Kajornwongwattana

Wipaporn Kajornwongwattana
Managing Editor
Interprofessional Journal of Health Sciences (Interprof. J. Health Sci.)
formerly Bulletin of Health, Science and Technology (BHST)
Rangsit University, Thailand

Detection of Influenza A Virus and Influenza B Virus Using Isothermal Reverse Transcriptase-Helicase Dependent Amplification Solana Influenza A+B Assay

Rujikorn kanlayanadonkit[1], Acharawan Thongmee[1*], Ekawat Prasomsub[2], Patamaporn Sukplang[1], Wanida Pongstaporn[1], Wasun Chantratita[2]

[1]Biomedical Sciences Graduate Program, Department of Medical Science, Faculty of Science, Rangsit University, Pathum Thani 12000, Thailand
[2]Department of Pathology, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok 10400, Thailand

Corresponding author: acharawan@rsu.ac.th

ABSTRACT

Influenza is a contagious respiratory tract infection usually caused by influenza A virus (Flu A) and influenza B virus (Flu B). Diagnosis of Influenza is very important in patient management and infection control. Therefore, rapid, low hands-on, and economically testing systems are needed, especially in influenza peak seasons. Currently, several laboratory methods are used for the detection of the Influenza virus, such as viral culture, serology, rapid antigen testing, and reverse transcription polymerase chain reaction (RT-PCR). The RT-PCR, the reference method for Influenza detection, is an accurate method but quite expensive, and it takes at least 4 hours to complete the test. The Solana Influenza A+B, an Isothermal Reverse Transcriptase-Helicase dependent amplification (RT-HAD) method, is suggested as a new method for Influenza testing with minimum turnaround time. This study aimed to evaluate the performance characteristics of the Solana Influenza A+B assay and compare the results to the RT-PCR.

A total of 260 samples of nasal swab and nasopharyngeal swab from patients with and without Influenza infections were tested in comparison with the reference method. The results showed that the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of the Solana Influenza A+B assay were 100% in all test parameters. In addition, Solana Influenza A+B assay can detect Influenza A virus and Influenza B virus as low as 1,080 copies/µl and 115 copies/µl, respectively. In addition, cross-reactivity was not observed when testing with other viral infected samples such as human rhinoviruses, respiratory syncytial viruses A, respiratory syncytial viruses B, coronaviruses, and meta-pneumoviruses. The Solana Influenza A+B assay and the RT-PCR displayed an excellent agreement for the detection of Flu A and Flu B. The Solana Influenza A+B assay was found to be a sensitive and fast alternative method for Flu A and Flu B detection in respiratory clinical samples.

Keywords: Solana Influenza A+B Assay, Influenza A virus, Influenza B virus

INTRODUCTION

Influenza, an acute respiratory tract infection commonly known as "Flu", is one of the most important infectious diseases since the emergence of a pandemic of this disease occur many times. Influenza has been recognized since the 16th century and spreads rapidly through communities in outbreaks. The clinical features of Influenza are sudden high fever, headache, muscle aches, and fatigue. Clinical manifestation of this disease ranges from asymptomatic to fulminant, depending on the characteristics of the virus and the individual host state of health. In addition, the complications of this disease include bacterial pneumonia, acute respiratory distress syndrome, encephalopathy, myocarditis, pericarditis, and myositis (Nayak et al., 2004; Lowen et al., 2007). Mortality for seasonal influenza is greatest in the very young and the elderly, with more than 90% of deaths in 65 years of age and older patients (Fiore et al., 2010).

Influenza is caused by influenza viruses, enveloped single-stranded, negative-sense, segmented RNA viruses belonging to the family *Orthomyxoviridae*. Influenza virus, unique in its ability to change, causes annual epidemics and periodic pandemics of acute respiratory disease in people of all ages. Influenza viruses are classified into four distinct types; Influenza A virus, Influenza B virus, Influenza C virus, and Influenza D virus (Medina and García-Sastre, 2011; Qi et al., 2011; Taubenberger and Morens, 2010). The four virus types differ in host range and pathogenicity. Influenza A and Influenza B viruses contain eight discrete gene segments, each segment coding for at least one protein. They are covered with three protein projections; hemagglutinin (HA), neuraminidase (NA), and matrix 2 (M2). Each influenza RNA segment is encapsidated by nucleoproteins to form ribonucleotide nucleoprotein complexes. Influenza B virus and Influenza C virus are almost all isolated from humans. Influenza A virus derived from an avian reservoir can infect a wide variety of warm-blooded animals including humans, swine, horses, dogs, cats, and other mammals. Aquatic birds are the natural reservoir for many subtypes of influenza A virus and probably are the source of human pandemic influenza strains (Taubenberger and Morens, 2010). Influenza A virus has a reservoir in wild aquatic birds of 16 hemagglutinin

and nine neuraminidase subtypes that are a source of reassortment in nature and major antigenic shift. Point mutations during replication of both influenza types A and B lead to antigenic drift.

Clinical diagnosis of influenza is difficult because symptoms are varied and have common characteristics with other respiratory viruses. Signs and symptoms of Influenza such as fever and cough as well as patient's history and patient's community were usually used for clinical diagnosis. Moreover, the accuracy of clinical diagnosis is influenced by host's characteristics and the prevalence of influenza in the community. Laboratory investigations for this infectious agent aid in the diagnosis and can be used to guide treatment decisions, avoid inappropriate use of antibiotics, and provide information for influenza surveillance. Laboratory tests for Influenza virus include viral culture, viral antigen detection, immunofluorescence and nucleic acid testing (Paules and Subbarao, 2017). Viral culture is a gold standard method and is also used for vaccine production. Immuno-chromatography is a rapid method but the sensitivity of this method is low. The reverse transcription polymerase chain reaction (RT-PCR) provides high accuracy but is expensive and takes about 3-5 hours to complete (Landry, 2011). Detection of Influenza virus by RT-PCR shows reliability but it requires multiple diagnostic devices and high level equipment available only in large laboratories. Since the diagnosis of the Influenza is important for prompt treatment and the epidemic control, a rapid and effective detection method of Influenza virus needs to be developed to reduce morbidity and mortality in viral infected patients.

The Solana Influenza A+B Assay has been developed to address the shortcomings of the current commercial molecular devices while improving upon their benefits. It is used for determination of viral nucleic acids from the sample without the need for extraction. The assay consists of two major steps: 1) specimen preparation, and 2) amplification and detection of target sequences specific to influenza A and influenza B using Isothermal Reverse Transcriptase - Helicase-Dependent Amplification (RT-HDA) in the presence of target-specific fluorescence probes. This technology is less time consuming and requires small integrated device.

The objectives of this study are to evaluate the performance of Solana Influenza A+B Assay for the detection of Influenza A virus and Influenza B virus and compare the results with the RT-PCR reference method.

MATERIALS AND METHODS

Samples and Sample Preparation

Two hundred and sixty nasal swabs and nasopharyngeal swabs used in this study were kindly provided by the Virology Laboratory, Department of Pathology, Faculty of Medicine Ramathibodi Hospital. Two hundred and forty nasal swabs and nasopharyngeal swabs were obtained from suspected Influenza virus infection patients while twenty samples were obtained from other respiratory tract infections besides Influenza virus. The samples were stored at -20°C. The samples were thawed at room temperature before testing.

Solana Influenza A+B assay

According to the manufacturer's recommendations, fifty microliters of sample were added to 1.5 ml viral transport media (VTM) in the process buffer tube. The tube was then mixed in the vortex mixer for 5 seconds and incubated at 95°C for 5 minutes to break the cells. Then fifty microliters of the process buffer were added to the reaction tube containing a competitive process control (PRC) and the reaction tube was put into the Solana machine. At this step viral genetic material was amplified (Figure 1).

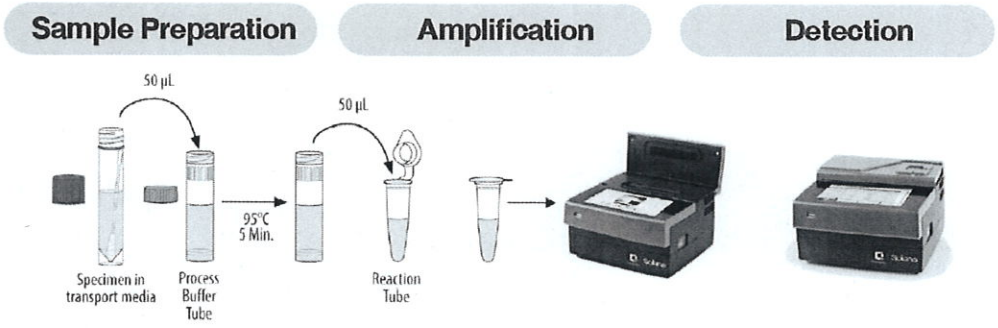

Figure 1 Solana Influenza A+B workflow

Solana processed for testing of Influenza A and Influenza B automatically. After the processed sample was transferred to a reaction Tube. The reaction tube contained lyophilized RT-HDA reagents, dNTPs, primers and probes. In the Solana instrument, the target sequences (Matrix Gene) were amplified by influenza A and influenza B specific primers and detected by influenza A and influenza B specific fluorescence probes, respectively. A competitive process control (PRC) was included in the Process Buffer Tube to monitor sample processing, inhibitory substances in clinical samples, reagent failure, or device failure. The PRC target was amplified by influenza B specific primers and detected by a PRC specific fluorescence probe. The two target probes and PRC probe were labeled with a quencher on one end and a fluorophore on the other end. In addition, the two target probes and PRC probe had one or more bases that were comprised of ribonucleic acid. Upon annealing to influenza A, influenza B or PRC amplicons, the fluorescence probes were cleaved by RNaseH2 and the fluorescence signal increased due to physical separation of fluorophore from quencher. The entire reaction took place at 65°C. Solana measured and interpreted the fluorescent signal, using on-board method-specific algorithms. The positive results of the test indicated that the RNA detection of Influenza virus was possible and the negative results indicated that there was no viral RNA detected, while the PRC was detected. The device displayed invalid only when Influenza A virus, Influenza B virus, and PRC were not detected (Solana® Influenza A+B Assay; Quidel Corporation, OH, USA)

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed following the procedure of Lyra Influenza A+B assay, while the RNA extraction procedure was performed using the EASYMAG extraction kit. Briefly, Influenza viral RNA was extracted from nasal swabs and nasopharyngeal swabs using BOOM technology in the eMAG instrument (Biomerieux Italia SpA, Italy). Two hundred microliters of sample were mixed with 1000 µl lysis buffer into the vessels. The mixture was well mixed up and down by pipette, then incubated at room temperature for 10 minutes. Then the extracted RNA was dissolved in the vessel and carefully transferred to new tubes and stored at - 20 ◦ C until used for real-time RT-PCR.

Mastermix preparation for the Lyra real-Time PCR Influenza A+B detection kit contained 15 µl of rehydration of lyophilized master mix (Buffer, Mg^{2+}, dNTP, and *Taq* polymerase) and 5 µl of nucleic acid template or internal positive control or internal negative control. The Real-Time

PCR conditions were the initial reverse transcription at 55 ºC for 5 minutes, at 60 ºC for 5 minutes and at 65 ºC for 5 minutes followed by amplification at a temperature of 92 ºC for 5 seconds, at 57 ºC for 40 seconds, at 92 ºC for 5 seconds and at 57 ºC for 40 seconds. Then it was entranced to PCR cycling for 35 cycles at 92 ºC for 5 seconds and at 57 ºC for 40 seconds. The RNA was amplified using Lyra Real-Time PCR (Lyra® Influenza A+B Assay; Quidel Corporation, OH, USA).

Diagnostic performance evaluation

Influenza A virus and Influenza B virus detection results obtained from the Solana Influenza A+B assay were compared to the results from the Real-Time RT-PCR reference method. In this study Lyra real-Time PCR Influenza A+B detection test US-FDA approved was used as the Real-Time RT- PCR reference method

The sensitivity or the limit of detection was evaluated by diluting the positive control sample (Amplirun total Flu A/Flu B/RSV) containing Influenza virus type A or B and then determining for the lowest value that can be distinguished from the absence of virus. In this study, the sensitivity was determined by using Influenza A virus and Influenza B virus concentration starting at 1,800 copies/µl and 2,300 copies/µl, respectively, for serial dilutions of virus ranging from 23-1800 copies/µl for Influenza A virus and ranging from 28-2,300 copies/µl for Influenza B virus.

The specificity determination or cross-reactivity evaluation was determined by testing with 20 non Influenza virus type A or B samples such as Human rhinoviruses, Human respiratory syncytial viruses A, Human respiratory syncytial viruses B, Human coronaviruses 229E, Human coronaviruses OC43, and Human metapneumoviruses.

RESULTS

A total of 260 samples were examined for Influenza A and B virus using Solana influenza A+B and Real-Time PCR as a reference method. The results revealed that 91 samples and 99 samples were positive for Influenza A and Influenza B, respectively, while 70 samples were negative for both Influenza A and Influenza B as showed in Table 1.

Table 1 The results of 260 samples tested with Solana influenza A+B and Real-Time PCR reference method for calculated sensitivity and specificity.

Virus	Clinical Results	Number (samples)
Flu A	Flu A positive with both Real-Time RT-PCR and Solana influenza A+B	91
	Flu A negative with both Real-Time RT-PCR and Solana influenza A+B	70
	Flu A Positive with Solana influenza A+B only	0
	Flu A Positive with Real-Time RT-PCR reference method only	0
Flu B	Flu B positive with both Real-Time RT-PCR and Solana influenza A+B	99
	Flu B negative with both Real-Time RT-PCR and Solana influenza A+B	70
	Flu B Positive with Solana influenza A+B only	0
	Flu B Positive with Real-Time RT-PCR reference method only	0
	Total	260

Twenty samples containing any viruses other than Influenza A and Influenza B virus were tested using Solana Influenza A+B isothermal amplification method for cross-reactivity test. The results showed that Influenza A and Influenza B virus were not detected in those samples by Solana Influenza A+B isothermal amplification method as showed in Table 2.

Table 2 The Solana Influenza A+B results of other different 20 virus strains

Clinical Results	No. of virus strain	Solana Influenza A+B
Human rhinoviruses	4	Not Detected
Human respiratory syncytial viruses A	3	Not Detected
Human respiratory syncytial viruses B	3	Not Detected
Human coronaviruses 229E	4	Not Detected
Human coronaviruses OC43	4	Not Detected
Human metapneumoviruses	2	Not Detected
Total	20	

The limit of detection (LOD) of the Solana influenza A+B was tested by serial dilution of Flu A and Flu B positive control samples staring concentration of 1,800 and 2,300 copies/reaction, respectively. It was found that the minimum detectable viral copy for Flu A and Flu B were 1,080 copies/μl and 115 copies/μl, respectively, as shown in Table 3

Table 3 Quantitative data of limited of detection

Positive control (µl)	Transport media (µl)	Influenza A (copies/µl)	Influenza B (copies/µl)	Result Influenza A	Result Influenza B
50	0	1800	2300	Positive	Positive
40	10	1440	1840	Positive	Positive
30	20	**1080**	1380	**Positive**	Positive
20	30	720	920	Negative	Positive
10	40	360	460	Negative	Positive
5	45	180	230	Negative	Positive
2.5	47.5	90	**115**	Negative	**Positive**
1.25	48.75	45	57.50	Negative	Negative
0.625	49.375	23	28.75	Negative	Negative

Evaluation of the performance of the Solana influenza A+B revealed that there was no false positive and false negative. In addition, the sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of the Solana influenza A+B were 100% in all test parameters.

DISCUSSION

Influenza is one of the most important respiratory tract infections. Clinical manifestation of this disease ranges from asymptomatic to fulminant. In addition, the complications of this disease lead to other viral and bacterial pneumonia and acute respiratory distress syndrome which can cause high mortality rate especially in very young and elderly people (Nayak et al., 2004; Lowen et al., 2007). Clinical diagnosis of influenza is difficult because symptoms are varied and have common characteristics with other respiratory viruses

In the diagnosis of infectious diseases, clinical manifestations often take precedence, but sometimes similar clinical manifestations is not enough to provide an accurate diagnosis. Therefore, additional laboratory examinations are required. If a simple diagnostic tool is

effectively used at the point of care providing results in a short time, it will be very useful for the treatment. The Solana Influenza A+B assay is considered as a point-of-care testing (PoCT) which provides a rapid test result and can be done outside the sophisticated central laboratory (Moore 2013; Hongmei, et al., 2018). The rapid diagnosis of influenza may help in treatment and management of patients and lower overall treatment costs. The Solana Influenza A+B assay is a rapid diagnosis assays and is relatively simple to perform which can produce results within 40 minutes. In addition, this study suggested that Solana influenza A+B assay has high sensitivity and specificity compared to Lyra Influenza A+B Assay (Real-Time PCR). No false negative and positive results were found. Therefore, test results obtained from the Solana Influenza A+B assay were substantially comparable to Real-Time PCR. The Solana Influenza A+B assay takes only 40 minutes to complete the test while Real-Time PCR takes more than 4 hours.

The Solana Influenza A+B assay amplifies and detects the target sequences, Matrix gene, specific to Influenza A or Influenza B using Isothermal Reverse Transcriptase-Helicase-Dependent Amplification (RT-HDA) in the presence of target-specific fluorescence probes which is performed in the Solana instrument. In addition, Influenza A and Influenza B virus were not detected in 20 samples containing any viruses other than Influenza A and Influenza B virus when tested with Solana Influenza A+B isothermal amplification method. Therefore, this method showed no cross reaction with viruses other than Influenza A and Influenza B virus.

CONCLUSION

In conclusion, the commercially available in vitro diagnostic Solana Influenza A+B assay shows a high potential tool for the detection of seasonal influenza compared to Lyra Influenza A+B assay (Real-Time PCR kit). It is one of the attractive platforms for a clear diagnosis of Influenza. The assay detects viral nucleic acids from patient samples. A multiplex reaction is carried out under optimized conditions in a single tube for each of the detection of the target viruses present in the sample. The advantages of using Solana Influenza A+B are its low cost, reduced sample extraction process, and low turn-around time. It only takes 40 minutes compared to real-time PCR, which takes at least 4 hours to complete.

ETHICS STATEMENT

This study was approved by the Ethical Clearance Committee on Human Right Related to Research Involving Human Subjects Faculty of Medicine Ramathibodi Hospital, Mahidol University. The protocol number is ID 1303.

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