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Comparison of Molecular Assays for the Rapid Detection and Simultaneous Subtype Differentiation of the Pandemic Influenza A (H1N1) 2009 Virus

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In April 2009, the H1N1 pandemic influenza virus emerged as a novel influenza virus. The aim of this study was to compare the performances of several molecular assays, including conventional reverse transcription polymerase chain reaction (RT-PCR), two real-time reverse transcription (rRT)-PCRs, and two multiplex RT-PCRs. A total of 381 clinical specimens were collected from patients (223 men and 158 women), and both the Seeplex RV7 assay and rRT-PCR were ordered on different specimens within one week after collection. The concordance rate for the two methods was 87% (332/381), and the discrepancy rate was 13% (49/381). The positive rates for the molecular assays studied included 93.1% for the multiplex Seeplex RV7 assay, 93.1% for conventional reverse transcription (cRT)-PCR, 89.7% for the multiplex Seeplex Flu ACE Subtyping assay, 82.8% for protocol B rRT-PCR, and 58.6% for protocol A rRT-PCR. Our results showed that the multiplex Seeplex assays and the cRT-PCR yielded higher detection rates than rRT-PCRs for detecting the influenza A (H1N1) virus. Although the multiplex Seeplex assays had the advantage of simultaneous detection of several viruses, they were time-consuming and troublesome. Our results show that, although rRT-PCR had the advantage, the detection rates of the molecular assays varied depending upon the source of the influenza A (H1N1)v virus. Our findings also suggest that rRT-PCR sometimes detected virus in extremely low abundance and thus required validation of analytical performance and clinical correlation.

Keywords: Influenza virus infection, real-time PCR, multiplex PCR, RT-PCR

Influenza pandemics are rare but recurring events. In April 2009, the H1N1 pandemic influenza virus [influenza A (H1N1)v virus] emerged as a novel influenza virus and spread rapidly in the human population [14]. Owing to its superior sensitivity, molecular detection has become one of the mainstays of diagnostic virology. As such, several pandemic H1N1 detection assays have been established, including rapid immunodiagnostic tests, microarrays, and real-time reverse transcription polymerase chain reaction (rRT-PCR) [13, 16–19]. PCR-based methods are advantageous in that they can serve as both screening and confirmation tests, yielding a final result within a few hours. Among the current laboratory diagnostic tests, rRT-PCR, which determines virus copies by monitoring fluorescence accumulations, has many advantages over other detection techniques [8, 12]. After the emergence of the pandemic influenza A (H1N1)v virus in April 2009, PCR assays designed for specific detection of this novel virus variant were communicated internationally almost instantly. In addition, only RT-PCR-based detection methods allow rapid detection of influenza A (H1N1)v virus, and protocols for these methods were published very quickly. In fact, several rRT-PCR assays have been developed recently to detect the pandemic influenza A (H1N1)v virus [2, 4, 7, 15]. Although these assays are effective, they have some limitations. First, these assays are incapable of simultaneously typing and subtyping seasonal influenza viruses. Second, these rRT-PCR assays only target the novel H1 gene of the pandemic influenza A (H1N1)v virus [7, 20]. Therefore, multiplex PCR methods were developed with the goal of simultaneously detecting a panel of viruses [6]. The Seeplex RV7 assay and Seeplex FluA ACE Subtyping assay (Seegene Inc., Seoul, Korea) are based on a multiplex PCR method using dual priming oligonucleotides (DPO). The Seeplex Respiratory Virus Detection assay system was also introduced recently. The Seeplex RV7 assay uses two separate primer mixes and is capable of detecting seven types of RNA respiratory viruses (Seeplex RV7 assay); the Seeplex FluA ACE Subtyping assay is capable of detecting four subtypes of

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RNA influenza A virus. We evaluated these commercially available molecular assays that were used at the same time in Korea for their ability to detect this virus based on the publicly released hemagglutinin (HA) sequence (A/California/04/2009, GenBank Accession No. FJ966082). Protocols developed by the World Health Organization (WHO) Collaborating Center and the WHO H5 Reference Laboratory were used to test the conventional reverse transcription (cRT)-PCR and two rRT-PCR assay methods. The manufacturer instructions were used to test the Seeplex FluA ACE Subtyping and the Seeplex RV7 assays.

The aim of this study was to compare the performance of several molecular assays, including conventional reverse transcription PCR (cRT-PCR), two rRT-PCRs, and two multiplex RT-PCRs.

Materials and Methods

Subjects

Nasopharyngeal aspirate, combined throat and nasal swabs, nasopharyngeal swabs, and throat swabs were obtained from individuals fulfilling both clinical and epidemiological criteria for suspected influenza A (H1N1)v virus infection. Specimens were placed in viral transport media and transported to the laboratory within 24 h of collection. A total of 381 clinical specimens were collected from patients (223 men and 158 women) between September 2009 and March 2010 and were tested with both the Seeplex RV7 assay and rRT-PCR within a week after collection. We used the RNA from samples that were positive for influenza A (H1N1)v virus by Seeplex RV7 or rRT-PCR, but not both, in the other detection methods. This study was approved by the institutional review board of Chung-Ang University.

Isolation of RNA

Total RNA was extracted from specimens using an RNeasy mini kit with QIAshredder according to the manufacturer's instructions (Qiagen, Germany). The sample volume used for each extraction ranged from 50 to 350 µl (depending on availability), and the extracted RNA was resuspended in 30 µl of sterile distilled water as described previously [10].

Real-Time RT-PCRs

Protocol A, which is from the WHO Collaborating Center for Influenza at the Center for Disease Control (Atlanta, GA, USA), was followed without modification and performed using the Advansure Influenza A/H1N1 Real-Time PCR system (LG Life Science, Seoul, Korea). Protocol B was developed by the WHO H5 Reference Laboratory (Virology Division, Center for Health Protection, Hong Kong SAR, China) using a LightCyclerTM2.0 system (Roche Applied Science, Basel, Switzerland).

Conventional RT-PCR

The reverse transcription step was carried out using a RevertAid First Strand cDNA Synthesis Kit (Fermentas, Ontario, Canada) to synthesize cDNA. The RT-PCR assays were performed using the Seeplex FluA/NewH1N1 ACE Detection kit (Seegene Inc., Seoul, Korea).

Multiplex RT-PCRs

The clinical specimens were tested for respiratory viruses using the Seeplex RV7 assay for seven major respiratory viruses [influenza virus types A, including influenza A (H1N1)v virus, and B; parainfluenza virus types 1, 2, 3; respiratory syncytial virus; metapneumovirus; rhinovirus; coronavirus; adenovirus]. The clinical specimens were tested for four subtypes of influenza A virus using the Seeplex Flu ACE Subtyping assay. Target pathogens included influenza A virus, human influenza A virus subtype H1, human influenza A virus subtype H3, avian influenza A virus subtype H5,

Table 1. Targets for the detection of respiratory viruses and influenza A subtype by multiplex PCR assays.

Multiplex RT-PCR	Target	Accession No.	Amplicon size (bp)
Seeplex RV7 assay			
Internal control	rbcl	AJ46297	719
Influenza virus type B	HA gene	NC_002207	649
Adenovirus	Pol gene	AC_000015	534
Metapneumovirus	F gene	EF589610	469
Respiratory syncytial virus A, B	RSV A: F gene	AY198175	401
	RSV B: F gene	AY526558	
Rhinovirus A	5' NTR	EF173414	340
Parainfluenza virus 1, 2, 3	HN gene	NC_003461	263
Influenza virus type A	M1 gene	CY037320	206
Flu ACE subtyping assay			
Internal control			
Human-H3	HA	CY037479	590
Human-H1	HA	CY038762	474
Avian-H5	HA	CY014272	327
Swine-H1	HA	CY039893	262
Influenza virus type A	M1 gene	CY037320	206

Table 2. Samples with positive results by only one assay method (including conventional reverse transcription PCR, two rRT-PCRs, and two multiplex Seeplex assays).

No. of specimens	Seeplex RV7 assay	Protocol A rRT-PCR	Protocol B rRT-PCR	Conventional RT-PCR	Flu ACE subtyping assay
9	Positive	Negative	Negative	Negative	Negative
5	Negative	Positive	Negative	Negative	Negative
21	Negative	Negative	Positive	Negative	Negative

and human influenza A virus subtype H1N1 variant. Primers were designed at the conserved sequence in the hemagglutinin gene (Table 1).

Statistical Analysis

Statistical analyses were performed using SPSS version 13.0. A value of $p<0.05$ was considered statistically significant, and values are expressed as mean $\pm$ standard deviation (SD).

RESULTS

A total of 381 clinical specimens were collected from patients (223 men and 158 women) between September 2009 and March 2010. Specimens were tested using both the multiplex Seeplex RV7 assay and protocol A or protocol B rRT-PCR within a week after collection. The concordance rate for the two methods was 83% (317/381), and the discrepancy rate was 17% (64/381). Thirty specimens were positive by the multiplex Seeplex RV7 assay only, whereas 34 specimens were positive by protocol A or protocol B rRT-PCR only. We used the RNA from samples that were positive in both of these two assays to perform the other methods.

Samples Only Testing Positive in the Multiplex Seeplex RV7 Assay

Using the RNA from the samples testing positive in the multiplex Seeplex RV7 assay alone, we performed cRT-PCR, two rRT-PCRs, and a multiplex Seeplex Flu ACE Subtyping assay. Among the 30 total samples, nine were positive by all of the other methods, and nine were negative by all of the other methods. Six samples were positive by all of the methods except the protocol A rRT-PCR. The remaining six samples had various patterns of positive results.

Samples Only Testing Positive in rRT-PCR

Using the RNA from samples testing positive only by protocol A or protocol B rRT-PCR, we performed cRT-PCR, the other rRT-PCRs, multiplex Seeplex RV7, and the Seeplex Flu ACE Subtyping assay. Of the 34 samples, 21 tested positive by protocol B rRT-PCR only, five tested positive by protocol A rRT-PCR only, and six were positive by all of the methods. The remaining two samples were negative according to the multiplex Seeplex RV7 assay only.

Overall Positive Results for All of the Molecular Assays

There were 64 samples with discrepant results in our study using matrix gene RT-PCRs. Among these, there were 35 samples with positive results according to only one assay, including nine samples that were positive only in the multiplex Seeplex RV7 assay, five samples that were positive only according to protocol A rRT-PCR, and 21 samples that were positive only according to protocol B rRT-PCR (Table 2). In this study, we defined a "true" influenza A (H1N1)v virus-positive specimen as positive by at least two assay methods. Fifteen samples were positive by all assay methods. The rest (14 samples) had diverse results. Twenty-seven of the 29 positive samples were detected by cRT-PCR and the multiplex Seeplex RV7 assay (93.1%); the multiplex Seeplex Flu ACE Subtyping

Table 3. Twenty-nine samples with positive results by at least two influenza A (H1N1)v infection detection methods.

No. of specimens	Seeplex RV7 assay	Protocol A rRT-PCR	Protocol B rRT-PCR	Conventional RT-PCR	Flu ACE subtyping assay
15	Positive	Positive	Positive	Positive	Positive
6	Positive	Negative	Positive	Positive	Positive
2	Positive	Negative	Negative	Positive	Negative
2	Positive	Negative	Negative	Negative	Positive
2	Negative	Positive	Positive	Positive	Positive
1	Positive	Negative	Positive	Positive	Negative
1	Positive	Negative	Negative	Positive	Positive
Positive rates (total no.: 29)	27/29 (93.1%)	17/29 (58.6%)	24/29 (82.8%)	27/29 (93.1%)	26/29 (89.7%)

assay was positive in 26 samples (89.7%). Protocol A rRT-PCR was positive for only 17 samples (58.6%), and protocol B rRT-PCR was positive for 24 samples (82.8%) (Table 3).

Discussion

In this study, the concordance rate for the multiplex Seeplex RV7 assay and rRT-PCR was 83%, and the discrepancy rate was 17%. We retested the 64 discrepant specimens using the five molecular assays. Of these, 15 specimens were positive according to all of the methods. The discrepancies were possibly due to the different specimen types and different collection periods, which might explain why our study found low concordance between assays. Our results showed that protocol A rRT-PCR missed six samples that tested positive by Seeplex RV7. Therefore, the positive rate of protocol A rRT-PCR (58.6%) was lower than that of protocol B rRT-PCR (82.8%). Lam *et al*. [11] also reported that rRT-PCR using the WHO Collaborating Center for Influenza at the Center for Disease Control protocol (Protocol A) failed to detect low concentrations of virus compared with other rRT-PCR methods [11]. These findings suggest that the detection rates of molecular assays depend upon the source of the influenza A (H1N1)v virus. In our study, an influenza A (H1N1)v virus-positive specimen was defined as a sample testing positive by at least two assays. The positive rates for all molecular assays were as follows: 93.1% for the multiplex Seeplex RV7 assay, 93.1% for cRT-PCR, 89.7% for the multiplex Seeplex Flu ACE Subtyping assay, 82.8% for protocol B rRT-PCR, and 58.6% for protocol A rRT-PCR (Table 3). The cRT-PCR assay was capable of achieving high detection rates, which makes it a good alternative for laboratories where facilities for rRT-PCR are not available; however, it is time-consuming and troublesome. Even though current multiplex PCR-based assays require further validation because of the high rate of false positives [1, 5], the multiplex Seeplex assays using the DPO system are highly specific for detecting the influenza A (H1N1)v virus [9]. High sensitivity and rapid turnaround time are advantages of rRT-PCR, but it does require high technical expertise and specialized equipment [3]. Nine specimens were positive by the Seeplex RV7 assay only, five samples were positive by protocol A rRT-PCR only, and 21 samples were positive by protocol B rRT-PCR only. It is possible that the viruses detected in the nine specimens positive by Seeplex RV7 only were influenza A virus but not influenza A (H1N1)v virus. In samples that were only positive by protocol A or protocol B rRT-PCR, the fragments were sequenced. The sequences obtained were confirmed as influenza A (H1N1)v virus using the GenBank (National Center for Biotechnology Information) database with the Basic Local Alignment Search Tool (BLAST). Among the five specimens positive by protocol A rRT-PCR only, one (20%) was confirmed as influenza A (H1N1)v virus; among the 21 specimens positive by protocol B rRT-PCR only, five (24%) were confirmed as influenza A (H1N1)v virus. In general, rRT-PCR assays were more sensitive than cRT-PCR. However, our results indicated that because rRT-PCR was too sensitive, it had low confirmation rates, which was different from another study [11]. The low confirmation rates may be due to false positives or the fact that stored rather than fresh specimens were sequenced. Additionally, the fact that the specimens confirmed by sequencing were positive by rRT-PCR only suggests that rRT-PCR can detect very low concentrations of virus, but this requires further verification. Moreover, our positive rates were not representative of true positive rates, because we could not confirm them with viral culture or other methods.

In conclusion, all the molecular assays examined in this study were capable of achieving high clinically positive rates. Our results show that multiplex Seeplex assays using the DPO system yielded highly successful detection rates for the influenza A (H1N1)v virus and had the advantage of simultaneously detecting several viruses; however, the results of this assay were not immediately available owing to the requirement of longer amplification times than those in rRT-PCR and an additional electrophoresis step. cRT-PCR also yielded a high detection rate but was time-consuming and troublesome. Even though rRT-PCR has the advantage of high detection rates and rapid turnaround time, the detection rates of this molecular assay depend upon the source of the influenza A (H1N1)v virus. Our findings also suggest that rRT-PCR sometimes detected virus in too low an abundance and thus requires validation of analytical performance and clinical correlation.

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