

# Tools to Detect Influenza Virus

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In 2009, pandemic influenza A (H1N1) virus (H1N1 09) started to spread quickly in many countries. It causes respiratory infection with signs and symptoms of common infectious agents. Thus, clinicians sometimes may miss the H1N1 patient. Clinical laboratory tests are important for the diagnosis of the H1N1 infection. There are several tests available, however, the rapid test and direct fluorescence antigen test are unable to rule out the influenza virus infection and viral culture test is time consuming. Therefore, nucleic acid amplification techniques based on reverse transcription polymerase chain reaction assays are regarded as a specific diagnosis to confirm the influenza virus infection. Although the nucleic acid-based techniques are highly sensitive and specific, the high mutation rate of the influenza RNA-dependent RNA polymerase could limit the utility of the techniques. In addition, their use depends on the availability, cost and throughput of the diagnostic techniques. To overcome these drawbacks, evaluation and development of the techniques should be continued. This review provides an overview of various techniques for specific diagnosis of influenza infection.

**Key Words:** Influenza virus, H1N1, RT-PCR, laboratory diagnosis

## INTRODUCTION

The 2009 pandemic influenza A (H1N1 09), a major cause of health burden, has gained worldwide attention of scientists for better diagnosis and treatment. It is one of the important causes of acute respiratory infection and causes disease in any age group. However, the infection rate is higher among infants and children.<sup>1-4</sup> It also accounts for other complications like pneumonia, viremia, myositis, myocardial dysfunction and encephalopathy.<sup>5</sup> The H1N1 09 spread in 214 countries with more than 18306 deaths.<sup>6</sup> Developing countries are comparatively at more risk because of lack of hygiene, overpopulation, poverty and scarcity of modern tools for the diagnosis and treatment. The influenza virus is known for continuous antigenic change through antigenic drift and shift.<sup>7</sup> This property of the virus has a significant effect on prevention (vaccination), diagnosis and treatment. Although an emergence of new strain of the virus cannot be stopped, its spread from the origin might be limited by preventing country to country transmission. It has been reported that more than 90.0% of cases of H1N1 endemic occurred in England by in-country transmission.<sup>4,8</sup> From the 2 pandemics (Severe Acute Respiratory Syndrome and

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H1N1 09) in the last decade, it is clear that rapid identification and proper surveillance tremendously help respond to the outbreak in short time, providing security to global public health.

One of the major preventions from circulating strains of influenza virus and the complications associated with the viral infection is by vaccination. As the virus is seasonal, the vaccine needs to be prepared according to the prediction that certain strain would circulate in a particular season.<sup>9</sup> Also, antiviral agents against influenza are found to be effective when used early in the course of illness. There are four drugs available against influenza virus: amantadine, rimantidine, zanamivir and oseltamivir.<sup>10</sup> Some studies have reported the resistance pattern of the H1N1 09 virus to amantadine and oseltamivir.<sup>11-13</sup> Rapid detection of such resistance is clinically important in reducing the time between diagnosis and initiation of individualized therapy. Therefore, close monitoring of the prevalence of antiviral resistance and discoveries of drugs and potent vaccines are necessary to control influenza burden.

Another important aspect of controlling influenza burden is early diagnosis. However, diagnosis by a physician is difficult because many of the respiratory viruses produce similar symptoms like influenza. Therefore, clinical laboratory diagnosis is mandatory for correct and early diagnosis. Although several tests have been developed to detect influenza viruses, they have different sensitivity, specificity, throughput, cost, availability, and other factors involved in from pre-analytical to post-analytical stages of clinical laboratory testing. These factors play a crucial role in the selection of diagnostic test method in the clinical laboratory. Use of highly sensitive and specific test to yield accurate result in relatively short time is beneficial to patients by reducing the adverse effects and complications with the use of appropriate drugs and antiviral therapy on time.<sup>14</sup>

## LABORATORY DIAGNOSIS OF INFLUENZA VIRUS

### Sample

For the influenza detection, nasopharyngeal (NP) swabs and NP aspirates are the best choice of samples.<sup>5</sup> To collect an aspirate, small amount of sterile saline is applied into the nose, and suctioned to collect the resulting fluid (saline and mucus). Then, the sample is put into a special container with preservatives for the organism for delivery to the laboratory.<sup>15</sup>

The NP swab is collected by inserting a Dacron swab into one of the nostrils until resistance is met (about 1 to 2 inches in), then rotated several times and withdrawn. Doctors usually use NP swabs on adults and nasal wash or aspirate on a child. In some situations, a doctor may use a throat swab, but this contains less virus than an NP aspirate, therefore, may not be suitable for use in rapid testing. For antibody testing, a blood sample is used.<sup>15</sup> Furthermore, the sample with long storage with repeated freezing and thawing has been found to degrade viral RNA or cDNA, and for samples with low viral copies, degradation may limit the quantitation by polymerase chain reaction (PCR) technique.<sup>16</sup> However, samples with high viral titres do not limit the quantitation after several freezing and thawing cycles.

## TESTS TO DETECT INFLUENZA VIRUS

### Serological diagnosis

Antibodies are produced after the onset of influenza virus illness and can be detected using serological diagnostic techniques like hemagglutination inhibition assay (HIA), enzyme immunoassay (EIA), complement fixation, and neutralization tests.<sup>9</sup> One study reports that the antibody titer reaches peak within 14 days of illness. They found more than 80.0% of reverse transcription polymerase chain reaction (RT-PCR) positive influenza virus to seroconvert by using the HIA or virus micro neutralization assay.<sup>17</sup> HIA is found to be useful to prohibit vaccinations when there is likelihood of severe reactions during the next outbreak of H1N1.<sup>17,18</sup> However, this method is not regarded as effective diagnostic tool because it is not a convenient method as it requires two serum samples with accurate timing and also has longer throughput.<sup>9</sup> Therefore, Centers for Disease Control and Prevention (CDC) does not recommend this technique for the diagnosis purpose. In addition, the test result is not interpretable with possibilities of misguidance in clinical decision making.<sup>19</sup>

### Viral culture

Although viral culture is time consuming, it has been the major technique to detect the influenza virus for many years.<sup>20</sup> For instance, monkey kidney cells, Madin Darby canine kidney (MDCK) cells and A549 cells are used to detect influenza viruses. After inoculation, the viruses cause cytopathology, which is different according to the cell type used. It takes around 10 to 14 days to get the result which delays ini-

tiation of antiviral therapy or infection control methods.<sup>9,21,22</sup> Human respiratory epithelial cells are also known to enhance the growth of influenza virus.<sup>23</sup> The shell vial culture (SVC) method was introduced in 1990s.<sup>24</sup> The combination of SVC and certain monoclonal antibodies have been found to decrease the turnaround time along with increased sensitivity and specificity.<sup>25</sup> On the other hand, a commercial R-Mix cell (Diagnostic Hybrids, Athens, OH, USA) is found to be more sensitive than the other cell culture techniques. It's a cell mixture containing Mink Lung epithelial cells and A549 cells. The use of this cell mixture can give result after 24-48 h of inoculation.<sup>26</sup> R-mix Too is another cell mixture of MDCK and A549 cells. It can detect respiratory viruses and amplifies their replication. In order to identify H1N1 09 specifically, the kit named D3 Ultra 2009 H1N1 Influenza A ID kit (2009 H1N1 ID kit; Diagnostic Hybrids, Inc., Athens, OH, USA) is developed which utilizes the R-mix Too cells. This method uses immunofluorescence method for the specific diagnosis of H1N1 09.<sup>27</sup>

Although the viral culture method for the detection of influenza virus is adopted in many clinical settings, newly available rapid tests and PCR-based tests are gaining popularity for their fast detecting ability with comparatively more sensitivity and specificity. Rapid tests are known to have 50.0-70.0% sensitivity in comparison to viral culture method or PCR-based methods.<sup>28</sup> One study revealed a similar sensitivity and specificity between rapid antigen test and viral culture technique, however, the PCR-based assay had more sensitivity and specificity than other tests.<sup>29</sup> The specificity of the rapid tests has been found to be 90.0-95.0%.<sup>14</sup> As the rapid tests results depend on the viral titer, a sample with lowest virus titer should be processed with the PCR-based assays. Most importantly, PCR technique has been found to yield high sensitivity and specificity in adults who have low titre of viruses than children.<sup>30</sup>

## RAPID INFLUENZA DETECTION TESTS (RIDTS)

Every patient and clinician wish to know the causation of disease as early as possible after the illness begins. To meet this aim, rapid tests to detect influenza virus have evolved. In the past decade, several tests have been developed and the use of rapid tests is found to flourish day in day out. This is true because of their ability to provide test results within about half an hour with easy interpretability. Although these

tests have not been able to gain trustworthiness because of several limitations like frequent false positivity and false negativity, they are known to yield better results with sample having higher titer, as in children than in adult cases.<sup>31</sup> Especially in developing countries where the PCR technique is not available in primary health care settings, they aid in clinical decision making and have been found to be used solely for diagnostic purpose. These tests are handy and also known as near patient or point of care testing methods. Today's focus on rapid identification of influenza should be centered to increase the sensitivity and specificity of these tests, so that they can be of potential use in early and accurate diagnosis of influenza.

The commercially available rapid antigen tests are mostly immunoassays that target the antigen of influenza virus. However, they have relatively poor sensitivity in comparison to virus culture technique and PCR-based assays. In one study using QuickVue Influenza A+B (Quidel) with reference as RT-PCR, they found the sensitivity and specificity for the detection of H1N1 09 was 20.0% and 99.0%, respectively, suggesting the use of RT-PCR for the clinical diagnosis.<sup>29</sup> Other studies have, however, found its sensitivities up to 51.0% and 53.3% in comparison to RT-PCR.<sup>32,33</sup> In another study using two rapid antigen tests, BD Directigen EZ flu and TruFlu, Meridian, they got a better performance with former kit than the later one. The sensitivity and specificity for H1N1 09 detection with BD Directigen EZ flu was 20.6% and 99.0%, whereas those of TruFlu, Meridian were 9.7% and 98.2%, respectively.<sup>34</sup> Tsao, et al.,<sup>35</sup> recently analyzed four rapid tests, namely BinaxNow test, QuickVue test, TRU test, and Formosa Rapid test, and found their detection limit to be lower than that of RT-PCR. Nevertheless, they found the sensitivity of Formosa Rapid test to be similar to QuickVue test, relatively more sensitive than the other 2 kits for H1N1 09 diagnosis. Furthermore, another study using SD Bioline Influenza Antigen Test<sup>®</sup> kit found a poor performance, with sensitivity and specificity of 44.0% and 99.9%, respectively, for H1N1 09 diagnosis.<sup>36</sup> In contrast, a recent study has revealed 90.0% sensitive Actim Influenza A&B kit (Medix Biochemica, Joensuu, Finland) for the detection of influenza A virus among 1-3 years old children.<sup>37</sup> The test performed with QuickVue influenza test in 6 months to 14 years of children showed 67.5% sensitivity and 96.0% specificity for influenza viruses.<sup>38</sup> This indicates that pediatric group have generally higher viral load fair enough for the kits to detect the virus in comparison to adults or other patients. The next important thing about the rapid

kits is that their sensitivity and specificity are inconsistent.<sup>31</sup>

## IMMUNOFLUORESCENCE TESTS

In comparison to PCR-based assays, direct fluorescence antibody assay (DFA) confers cheap and fast result, although it is not as sensitive as the PCR test. Several studies have been performed, especially surveillance ones, solely based on the DFA test.<sup>39</sup> The technique involves the staining of cells from the sample, followed by bioconjugation of antibodies to the fluorescent dye. The time frame to obtain the results is between 2 and 4 h.<sup>40,41</sup> The sensitivity of the DFA tests for influenza virus is found to be between 70.0% and 100.0%. Similarly, specificity ranges from 80.0% to 100.0%,<sup>22</sup> indicating that the sensitivity is comparatively higher than that of rapid antigen detection kits and lower than the PCR tests. This means that the negative results need to be confirmed by the PCR-based assays. Although some studies recommend the use of DFA as well as SVC together, to increase the sensitivity of the test by 5.0-15.0% than the DFA alone, multiple virus infections cannot be ruled out.<sup>22</sup> Recent study has shown that, particularly for H1N1, the sensitivity and specificity of DFA were 62.0% and 100.0%, respectively, when compared to RT-PCR, with variability in age. The performance of the test was better with children below 10 years of age, whereas the sensitivity of the test decreased with persons above 30 years of age.<sup>42</sup> In contrast, the sensitivity of DFA was 93.0% with adult in a study by Pollock, et al.,<sup>41</sup> in 2009 for H1N1 09. In another study, the sensitivity of DFA is shown to be lowered to 44.4% in comparison to RT-PCR for H1N1.<sup>43</sup> Similarly, the study comparing DFA, rapid antigen test and RT-PCR showed that the former two tests have comparatively low sensitivity (38.7% and 18.2%, respectively) in comparison to RT-PCR, suggesting the confirmatory test as RT-PCR for H1N1 09.<sup>44</sup>

However, the DFA test requires skilled expertise for fluorescence microscopy. Furthermore, the DFA test is carried out in shift basis generally in the clinical settings, which prolongs the turnaround time. Thus, it does not seem to be a suitable test in regard to clinical laboratory diagnosis of influenza virus in comparison to PCR based assays.

## MOLECULAR METHODS

Number of studies have reported that PCR-based assays

overcome several demerits possessed by other techniques such as rapid influenza detection tests (RIDTs) and SVC with respect to sensitivity, specificity and time frame.<sup>45,46</sup> For both qualitative and quantitative approach of diagnosis, the RT-PCR is regarded as the king of diagnosis for the viruses, including influenza virus. There are 2 types of RT-PCR. One step RT-PCR involves the use of oligo-dT or random primers for reverse transcription. Next is two step RT-PCR where reverse transcription is performed and then PCR is carried out. Briefly, RNA extracted from the influenza sample is purified and transcribed using the oligonucleotides specific to the target sequence, producing cDNA.<sup>47</sup>

The first PCR-based assay in detecting influenza virus was described in 1991 by Zhang and Evans.<sup>48</sup> Since then, many modifications and highly sensitive methods based on PCR have been developed. For instance, PCR-enzyme immunoassay has been found to have better performance than the culture method in cases with 5-9 days of illness.<sup>49</sup> Similarly, RT-PCR followed by electrospray ionization mass spectrometry is able to detect the culture-unidentified viruses.<sup>50</sup> When the H1N1 outbreak occurred in 2009, CDC recommended the use of one step RT-PCR technique targeting the matrix gene of H1N1 09.<sup>51</sup> The type and subtype specific detection methodology using this technique have been already described using the hemagglutinin gene as target in a single tube, which minimized the cost and time required to analyze the sample for each type and subtype of the influenza virus.<sup>49</sup> Other PCR-based assays such as ResPlex II (Qiagen, Hilden, Germany), MultiCode PLx RVP (EraGen Biosciences, Madison, WI, USA), Seeplex RV (Seegene Inc., Seoul, Korea), NGEN RVA ASR (Nanogen Inc., San Diego, CA, USA), xTAG RVP (Luminex Molecular Diagnostics, Toronto, Ontario, Canada), ProFLu+ (Prodesse Inc., San Diego, CA, USA) and ProFLu-1 (Prodesse Inc., San Diego, CA, USA) are also available.<sup>22</sup> Among them, the latter three are approved by Food and Drug Administration (FDA) for the detection of respiratory viruses, including influenza viruses. These multiplex RT-PCR techniques are highly sensitive and specific to give result in comparatively less time than the viral culture and EIA techniques.

Considering the difference between PCR-based assays and other techniques such as RIDTs and SVC in the aspect of merits of diagnosis of influenza virus, PCR method seems to be effective. However, intensive research in medical diagnosis has led even more effective diagnostic tools, based on PCR assays. For instance, nucleic acid sequence-based amplification tests are known to be more sensitive than the



RT-PCR tests for the detection of influenza virus. In this technique, three enzymes are used, namely AMV-RT, T7 RNA polymerase and RNaseH. It can detect the H1N1 09 in 90 min with sensitivity and specificity of 100.0%.<sup>52</sup> Moreover, multiplex nucleic acid-based amplification test along with an enzyme-linked oligonucleotide capture has shown to rapidly and specifically detect a single virus, including influenza viruses A and B from a multiplexed group, reducing laboratory testing time and enabling high throughput screening.<sup>53</sup>

Although nucleic acid-based amplification assays are found to yield better sensitivity in comparison to other methods, more other techniques are being studied by FDA. For instance, Verigene<sup>®</sup> Respiratory Virus Plus Nucleic Acid Test (RV+) (Nanosphere, Chicago, IL, USA), which is recently cleared by FDA, is an automated method for H1N1 09 diagnosis with the sensitivity and specificity of 99.5% and 100.0%, respectively.<sup>54</sup> Accordingly, Cepheid Company (Sunnyvale, CA, USA) developed a Flu A (Influenza virus) Panel test which is an FDA-cleared fully automated technique for the detection in 45 min.<sup>55</sup>

## DIAGNOSIS OF DRUG RESISTANT INFLUENZA INFECTION

The knowledge on the genetic basis of resistance to drugs has provided molecular techniques to quickly detect the mutations conferring resistance to the drugs. For instance, one of the most widely used drugs against influenza infection, oseltamivir resistance, arises due to histidine to tyrosine substitution in the neuraminidase active site (H275Y).<sup>56</sup> The techniques available to monitor the drug resistance include neuraminidase inhibition assay, pyrosequencing, use of genotype specific primers, or real time RT-PCR,<sup>56</sup> all of which have been known to be time consuming. Sequencing methods such as pyrosequencing provide detailed genetic information about the resistance pattern and also estimate relative proportion of variant and wild-type viruses in a mixed population sample and can be applied directly in clinical setting.<sup>57</sup> However, they need additional steps after initial PCR, specialized person to operate the sequencing instruments and the softwares.<sup>24</sup> Real-time quantitative RT-PCR provides a rapid, highly sensitive, and specific alternative to sequencing.<sup>24</sup> However, it is either specific for seasonal influenza A or needs multiple primers to detect H1N1 09.<sup>56</sup> RT-PCR, combined with pyrogram techniques, has provided the detection of H275Y sequences in H1N1 09

within 8 h with sensitivity similar to pyrosequencing method for seasonal H1N1.<sup>56</sup> Similarly, cycling probe real-time PCR assay has been shown to provide test results within 3 h after receiving the samples for resistance to oseltamivir and also to be able to detect both seasonal as well as H1N1 09 viruses resistant to oseltamivir.<sup>58</sup> However, the major drawback is that new sets of primers and probes have to be developed in the event when a novel drug-resistant strain would emerge during treatment.<sup>58</sup>

## CONCLUSION

Influenza virus is a significant global health burden. Timely diagnosis and initiation of treatment reduce the stigma of the disease, and also helps in outbreak control. Sole interpretation by a clinician, based on the symptoms presented by a patient, is not specific, as the virus has symptoms common to other known respiratory viruses. Therefore, clinicians are always in need of a confirmatory test for the influenza infection as soon as the illness initiates. The culture methods are time-consuming and are not as sensitive and specific as to make clinicians to start with antiviral therapy. However, they are still being used in many laboratories, suggesting that the influenza diagnosis by culture methods needs substantial quality check and continuous monitoring to increase the quality of report. The fast and specific RIDTs, also called as point of care tests, are also not appropriate for the diagnosis of influenza virus because of their poor performance. However, they are simple, cheap, easily interpretable and handy. If they had been showing similar or better performance than that of PCR-based assays, they would have been the major technique to detect the influenza virus. However, they too need to be studied well and improved to the extent where they can be used for accurate and specific diagnosis of the virus.

Recent advancement in the PCR-based assays have become popular in advanced countries. However, we cannot deny the fact that these popular models also have several limitations, ranging from nucleic acid extraction to result interpretation. Even a negligible amount of error has potential effect on the result interpretation of the highly sensitive tests. Therefore, they have also higher possibilities of yielding false positive results in comparison to viral culture methods. Thus, validation of reagents, application of proper positive and negative controls, and use of automation could reduce the chances of errors in molecular diagnosis of influ-

enza infection. On the other hand, cell culture methods and RIDTs should undergo standardization, quality assurance and quality control measures in order to validate the results. Hence, further studies are needed to validate the interpretation of influenza virus diagnostic techniques. Besides many merits and demerits, PCR based-assays are so far suggested technique for the diagnosis of influenza virus. Therefore, clinicians should rely on the RT-PCR test results of patient and correlate with the clinical aspects of the patient.

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