Rapid Identification of 2009 H1N1 Influenza A Virus Using Fluorescent Antibody Methods

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Key Words: 2009 H1N1 virus; Immunofluorescence; Culture; Direct specimen testing

Abstract

Identification of the 2009 H1N1 influenza A virus requires emergency use authorized (EUA) molecular reverse transcriptase–polymerase chain reaction. Laboratories lacking molecular capabilities outsource testing, which is costly and may delay result reporting. A fluorescent antibody (FA; D³ Ultra 2009 H1N1 influenza A virus ID Kit, Diagnostic Hybrids, Athens, OH) recently received Food and Drug Administration EUA status for 2009 H1N1 virus identification. The performance of this FA reagent was evaluated in this study.

Influenza A–positive nasopharyngeal specimens (seasonal H1, H3, and 2009 H1N1) were prepared for culture and FA testing and were stained using influenza A antibodies and the 2009 H1N1 reagent. Other respiratory viruses were also evaluated.

The FA reagent demonstrated 100% sensitivity and specificity. Bright, apple-green fluorescence was effortlessly identified in culture-positive cells, particularly around cell membrane perimeters. Laboratory-prepared slides were preferred over bedside-prepared specimens because background fluorescence obscured identification in the latter.

The new FA reagent provides an accurate, rapid, and inexpensive assay for identifying the 2009 H1N1 virus in nonmolecular diagnostic laboratories.
became approved by the Food and Drug Administration on an EUA basis. A blend of 2009 H1N1 virus antigen-specific murine monoclonal antibodies and a fluorescein-labeled conjugate is used to identify 2009 H1N1 viral antigen in culture and in direct respiratory specimens. Thus, laboratories lacking molecular diagnostic capabilities may use this new antibody reagent to rapidly and inexpensively identify 2009 H1N1 influenza A virus in house. This study evaluated the sensitivity and specificity of this novel reagent to identify 2009 H1N1 influenza A virus in shell-vial culture and directly from patient nasopharyngeal specimens.

**Materials and Methods**

**Specimens**

Study materials were obtained from remnant positive original nasopharyngeal specimens previously tested using rapid antigen detection, culture, and RT-PCR. Influenza specimens (50 influenza A/2009 H1N1; 10 influenza A/H3, 10 influenza A/seasonal H1, and 10 influenza B) were identified and subtyped using RT-PCR methods (Pro Flu and Pro Flu+, Gen Probe, San Diego, CA). Other respiratory pathogens (10 respiratory syncytial virus [RSV] specimens, 5 specimens each for parainfluenza types 1 through 3, 5 rhinovirus, 5 adenovirus, 3 enterovirus, and 10 human metapneumovirus [hMPV] training panel specimens) were also tested with the new FA reagent. Specimens were collected using mattress or flocked type swabs, placed in universal transport medium (Copan Diagnostics; Corona, CA), stored at 2°C to 8°C for 3 to 6 days, and thereafter kept frozen at −70°C.

**Shell-Vial Culture**

A total of 2 R-mix shell vials (A549/Mv1Lu cells; Diagnostic Hybrids) were inoculated with aliquots (0.1 mL) of original specimens and were centrifuged, incubated for 24 hours, and harvested according to the manufacturer’s prescribed protocol. Rhinovirus specimens were inoculated into MRC-5 shell vials. Monkey kidney shell vials were used to isolate enterovirus and hMPV samples (Diagnostic Hybrids).

**Direct Specimens**

A total of 25 microscope slides were prepared from original positive specimens (10 specimens of 2009 H1N1; 6 RSV specimens, 3 adenovirus specimens, 2 specimens each of parainfluenza 1 through 3) using a Cytospin 3 centrifuge (Thermo, Pittsburgh, PA). Additional manufacturer-prepared (Diagnostic Hybrids) direct specimen training panel slides (2 each) for RSV, influenza A and B, parainfluenza 1 through 3, adenovirus, and 10 negative preparations were also included for study. The hMPV training samples (Diagnostic Hybrids; 5 each of types 1A and B2) were prepared for examination as previously described for original patient specimens.

Direct bedside specimens were prepared by collecting posterior nasopharyngeal cells using mattress or flocked swabs. Cells were then transferred on glass slides at the bedside by rolling the swab on the slide surface. Positive slides for RSV (5) and influenza A2009 H1N1 (2) and 5 negative slides were stained with the 2009 H1N1 reagent. The low quantity of bedside prepared samples precluded their value in sensitivity and specificity analysis; however, they were used to contrast different direct-specimen preparation methods with fluorescent staining quality.

**FA Staining**

One shell-vial monolayer was stained with virus-specific antibodies (D³ Ultra respiratory ID kit, Diagnostic Hybrids) to confirm the presence of each virus type. hMPV and enterovirus were confirmed with hMPV (D³ Ultra Duet IF reagent, Diagnostic Hybrids) and D³ IFA-enterovirus (Diagnostic Hybrids) reagents, respectively. Rhinovirus was confirmed using a combination of 2 enterovirus FA reagents (D³ IFA-enterovirus and Light Diagnostics Panenterovirus blend, Millipore, Temecula, CA), as previously described.5

The second monolayer was stained with the new 2009 H1N1 reagent. Direct specimens were also stained and confirmed as described. Staining procedures followed specified manufacturer’s directions. I evaluated the specimens. Results were subsequently confirmed (positive or negative fluorescent activity) by the virology laboratory staff members who were blinded as to the specimen identities and the expected results.

Fluorescent microscopy was done using an episcopic microscope equipped with a 100-W mercury vapor light source, a 450- to 490-nm B-2A interference excitation filter, and a 515-nm barrier filter (Filter Unit B2A, Nikon, Garden City, NY). Specimens were examined at ×100 and ×400.

**Results**

**Shell-Vial FA Staining**

The results of FA staining are given in Table 1. The 2009 H1N1 reagent demonstrated 100% sensitivity and specificity when tested with 50 specimens of the 2009 H1N1 virus and 78 other virus specimens. Bright, apple-green fluorescence was demonstrated in positive cells, particularly around cell membrane perimeters. Staining of the cytoplasm...
and nucleus was also evident in some positive cells. Positive fluorescent activity was easily determined at low magnifications (×100). Similar fluorescent intensities were demonstrated between the kit’s control slides and patient samples. Negative cells demonstrated a red color from the addition of Evans blue dye added to the conjugate. Confirmation of all non–2009 H1N1 viruses occurred using the appropriate viral antibodies.

Direct Specimen Testing

The 2009 H1N1 reagent produced similar results when testing laboratory-prepared direct specimens. As expected, there were fewer positive cells observed in direct slides than in culture monolayers. Low and high magnifications were needed to confirm cellular fluorescence.

Background fluorescent staining was found in some laboratory-prepared slides; however, this artifact did not readily hinder result interpretation. Conversely, background fluorescence was pronounced in bedside-prepared specimens. The method used to prepare bedside specimens (rolling cells onto a glass slide) resulted in accumulating mucus on the specimen surface and prevented adequate cellular separation, which produced cellular clumping. Both of these factors seemed to increase the background fluorescence, making the bedside-prepared materials difficult to interpret.

Low columnar epithelial cells, abundant in the posterior nasopharynx, are infected by influenza virus and readily demonstrate fluorescent activity when appropriately tested with the appropriate antibody reagents. Determination of low columnar cell types was sometimes difficult in laboratory-prepared slides. Cellular clumping and dried mucus films present on bedside-prepared slides made determination of this cell type almost impossible. An evaluation of epithelial cell numbers was not presently done to evaluate specimen adequacy (acceptance based on ≥20 cells per slide).

Confirmation of non–2009 H1N1 viruses produced expected positive results when testing was done using their appropriate fluorescent antibodies.

| Table 1 |

Fluorescent Antibody Staining of Respiratory Virus–Positive Shell-Vial Cultures and Prepared Microscope Slides Using the D3 Influenza A 2009 H1N1 Reagent Kit

<table>
<thead>
<tr>
<th>Virus Type*</th>
<th>No. of Specimens† (Shell-Vial/Slide‡)</th>
<th>2009 H1N1 FA Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Influenza types</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/2009 H1N1</td>
<td>50/10</td>
<td>All positive</td>
</tr>
<tr>
<td>A/H1 seasonal</td>
<td>10/ND</td>
<td>All negative</td>
</tr>
<tr>
<td>A/H3 seasonal</td>
<td>10/2</td>
<td>All negative</td>
</tr>
<tr>
<td>Type B</td>
<td>10/05</td>
<td>All negative</td>
</tr>
<tr>
<td>Other viruses</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenovirus types (1, 2, 3, 5, 7)</td>
<td>5/5</td>
<td>All negative</td>
</tr>
<tr>
<td>Enterovirus types (coxsackie A9 and B2, echovirus type 9)</td>
<td>3/ND</td>
<td>All negative</td>
</tr>
<tr>
<td>Human metapneumovirus</td>
<td>10/10</td>
<td>All negative</td>
</tr>
<tr>
<td>Rhinovirus</td>
<td>5/ND</td>
<td>All negative</td>
</tr>
<tr>
<td>Parainfluenza types (1, 2, 3)</td>
<td>15/12</td>
<td>All negative</td>
</tr>
<tr>
<td>Respiratory syncytial</td>
<td>10/8</td>
<td>All negative</td>
</tr>
</tbody>
</table>

FA, fluorescent antibody; ND, not done.

* Viral presence was confirmed using FA techniques (adenoviruses, enteroviruses, human metapneumovirus, influenza A and B, parainfluenza, and respiratory syncytial virus).
† Two enterovirus antibody pools were used to identify rhinovirus, as described. Subtype analysis was done using neutralization techniques (adenoviruses and enteroviruses) and reverse transcriptase–polymerase chain reaction testing for seasonal and 2009 H1N1 strains of influenza A (Pro Flu, Gen Probe, San Diego, CA). Uninoculated cultures and negative slides served as control samples.
‡ Original respiratory specimens were obtained from patients (and placed in viral transport medium) and/or were obtained from culture and direct slide training panels (D3 Influenza A 2009 H1N1 Reagent Kit, Diagnostic Hybrids, Athens, OH).

Slides were prepared using a Cytospin 3 Centrifuge (Thermo, Pittsburgh, PA) from original specimens or were obtained from manufacturer-prepared direct-specimen training materials (Diagnostic Hybrids).
Discussion

The new 2009 H1N1 FA reagent offers the first EUA-cleared, nonmolecular tool to definitively identify the 2009 H1N1 virus. The presence of influenza A virus must be established in specimens, using an alternative approved method, before using the 2009 H1N1 reagent. A proposed laboratory flowchart for using this antibody reagent is shown in Figure 1. Specimen collection using flocked swabs over traditional mattress swabs is recommended because the former have demonstrated superior ability in collecting and releasing materials. Specimen collection is critical to the success of antigen detection methods.

Two R-mix shell vials are inoculated as described in the study design. If the first monolayer is influenza A-negative, the second monolayer can be examined using a pool of respiratory antibodies (ie, D3 Ultra screening reagent) to identify other common viruses.

Nasopharyngeal specimens may also be directly examined using antigen detection techniques. These assays are important in providing point-of-care testing in hospital emergency departments and physician offices on a 24-hour, 7-day basis. Despite poor sensitivity, positive results seem reliable and can be used to triage and manage hospital resource allocation. Positive influenza A specimens may then be tested using the new 2009 H1N1 antibody reagent to assess the need for patient isolation and appropriate care management with respect to particular 2009 H1N1 infection risks (eg, pregnancy and obesity).

Poor sensitivities demonstrated by enzyme immunoassay and chromatographic immunoassays for the 2009 H1N1 virus might necessitate retesting of negative specimens using FA techniques or shell-vial culture, if available. FA demonstrated greater sensitivity for 2009 H1N1 virus than other rapid immunoassay methods. Compared with PCR, FA negative predictive values of 96% or more were recently reported, also making it an effective test to rule out influenza A.

Direct specimens negative for influenza A may be additionally tested using a pool of respiratory antibodies that specifically identify a number of different respiratory viruses (D3 Ultra Duet respiratory ID kit, Diagnostic Hybrids). Result times of approximately 1 or 2 days are obtainable using culture. Direct testing can provide results within hours.

Shell-vial culture methods produced easily recognizable positive results at low (∼100) magnification. In facilities not equipped to perform culture, laboratory-prepared microscope slides using a Cytospin centrifuge provide rapid specimens with excellent morphologic detail. Cytospin slides are recommended over bedside-prepared materials because the former were easier to interpret and produced much less background fluorescence.

In hospitals lacking molecular diagnostics, outsourcing specimens for 2009 H1N1 testing can be costly (approximately $200 per test) and results are often delayed because of the needed time for triaging, handling, and transporting patient specimens. Our laboratory calculated the mean time from specimen collection to the availability of RT-PCR results to be 5.69 ± 0.37 days, offering little value to patient care.

Conclusion

The new 2009 H1N1 FA reagent demonstrated excellent sensitivity and specificity when tested by culture and direct immunofluorescent methods using a variety of respiratory viruses. It is important to note that the 2009 H1N1 reagent provides traditional, nonmolecular laboratories an inexpensive, rapid, and accurate in-house method to diagnose 2009 H1N1 virus, without expensive equipment and intensive staff training.

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References