Title Page

Title: Direct Fluorescent Antibody Followed by Culture for Diagnosis of 2009 H1N1 Influenza A

Running Title: DFA Followed by Culture for Diagnosis of H1N1

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Abstract:
During the 2009 H1N1 influenza A outbreak, 773 children were tested for influenza by direct fluorescent antibody with polymerase chain reaction confirmation. Direct fluorescent antibody has specificity of 99.6%, but sensitivity of only 65.0%. Physicians should recognize diagnostic limitations of direct fluorescent antibody which missed one third of infected individuals.

Manuscript
We retrospectively reviewed influenza testing data at a large tertiary urban pediatric hospital during the 2009 H1N1 influenza A outbreak. Patients were hospitalized, severely ill, or immunocompromised. Hospital protocol was to obtain nasopharyngeal swabs for direct fluorescent antibody (DFA) testing. If negative, viral culture was performed. All samples underwent polymerase chain reaction (PCR) testing for the 2009 H1N1 strain of influenza A. Diagnostic accuracy of the testing methods was compared for identification of this novel strain.

Samples were obtained between September 30 and December 1, 2009. Duplicate nasopharyngeal swabs were obtained simultaneously from a naris using Copan® nylon-tipped flocked swabs (Microrheologics Srl, Brescia, Italy). DFA was performed for multiple viruses. Sample swabs were directly applied to a microscope slide then placed into MicroTest M4-RT viral transport medium (Thermo Fischer Scientific, Lenexa, KS). Slides were stained with a D3 Ultra DFA Respiratory Virus Screening reagent (Diagnostic Hybrids, Athens, OH).
Positive samples were stained with influenza A specific fluorescein labeled monoclonal antibody (Light Diagnostics, Millipore Corp, Billerica, MA).

All DFA-negative samples underwent viral respiratory culture. Influenza virus culture was performed by inoculation of samples from the viral transport media into RMix shell and RhMK tubes (Diagnostic Hybrids, Athens, OH). RMix shell samples were stained with fluorescein labeled monoclonal antibody at 2 days. RhMK tubes were observed for hemoabsorption, and stained with fluorescein labeled monoclonal antibody.

Duplicate nasopharyngeal samples were sent to the Arizona state laboratory for 2009 H1N1 influenza A specific testing by real-time reverse transcriptase-PCR. PCR for 2009 H1N1 influenza A was performed using the World Health Organization Centers for Disease Control protocol. The assay utilized a panel of oligonucleotide primers and dual-labeled hydrolysis (Taqman®) probes for qualitative detection and characterization. The swInfA primer and probe set was used to detect swine influenza A virus (7).

Nasopharyngeal swabs were obtained from 773 children ranging from 5 days to 26 years of age. Median age was 3.04 years (5th, 95th %ile: 1.7 months, 15 years). Eighty-one percent (n=626) of the tested patients were hospitalized.
PCR identified 2009 H1N1 influenza A in 31.8% (n=246) of patients. DFA was positive in 162 patients, 160 of which were also PCR positive. DFA was negative in 611 patients, 86 of which were positive by PCR. This resulted in a sensitivity of 65.0%, specificity of 99.6%, positive predictive value (PPV) 98.8% and negative predictive value (NPV) 85.9%. Among those with a negative influenza DFA (n=611), 92.0% (n=562) underwent viral culture. Twenty-six cultures were not done despite negative DFA and 23 cultures were cancelled since DFA was positive for other viruses. Forty-two (7.5%) were culture positive for influenza A; the sensitivity, specificity, PPV, and NPV were 51.8%, 99.6% 95.6%, and 92.3%, respectively. Sequential testing (DFA positive or DFA negative/culture positive) increased sensitivity to 81.3% with specificity of 99.2%, PPV 98.0%, and NPV 91.9% (see table 1).

In 2 patients, viral culture was positive and PCR was negative; culture demonstrated typical cytopathic effect and was identified utilizing influenza A specific monoclonal antibody. In 2 patients, DFA was positive and PCR was negative; viral culture was not performed.

Reports of the diagnostic accuracy of DFA testing for diagnosis of 2009 H1N1 influenza A vary widely. There are case reports of false negative DFA tests even in severely ill adult patients with 2009 H1N1 influenza A and respiratory failure (5). In a study involving 112 primarily adult patients, DFA had sensitivity of 93%, specificity of 97%, NPV of 96%, and PPV of 95% relative to 2009 H1N1 influenza...
A specific PCR (6). In a larger study involving 6090 inpatients, outpatients and emergency department visits, DFA had a sensitivity of 47.2%, specificity of 99.6%, NPV of 90.6%, and PPV of 96.2% for the diagnosis of 2009 H1N1 influenza A. Age ranged from 4 days to 98 years, but authors did not differentiate between adult and pediatric populations (3). Another study involving 172 specimens reported DFA sensitivity of 38.7%, specificity of 100%, NPV of 82.2%, and PPV of 100% (2).

PCR is the most sensitive and specific test for the diagnosis of influenza and can differentiate between influenza serotypes (1, 4). However, this test may be too strain specific when multiple strains of influenza are circulating in the community. In 2 patients, viral culture was positive and PCR was negative. PCR was specific for 2009 H1N1 influenza A; positive culture may represent infection with non-H1N1 serotypes. False positive culture or false negative PCR are possible explanations, but are less likely. In 2 patients, DFA was positive and PCR was negative. These results may represent false positive DFA, false negative PCR or infection with a different strain of influenza A. DFA results are generally more difficult to interpret, making false positive DFA a possibility. Hospital protocol for viral testing did not require confirmation of positive DFA with culture, which could have clarified whether positive DFA represented infection with non-2009 H1N1 influenza A. The hospital protocol dictated that swabs were obtained and manually applied to slides prior to being placed in viral transport medium. While the purpose of this procedure is to accelerate rapid DFA testing, this procedure...
potentially introduces bias since test samples were not equally distributed for DFA culture and PCR.

This study reports DFA sensitivity of 65.0%; with addition of viral culture, sensitivity improved to 81.3% when compared to PCR as the gold standard. Both DFA and culture had excellent (>95%) specificity and positive predictive value. DFA alone missed 1/3 of infected patients later identified by PCR. Addition of viral culture increased diagnostic sensitivity, but results were not available rapidly. Over reliance on test results can lead to mis-diagnosis and lost opportunity for early initiation of therapy.
References:


7. World Health Organization. 2009. CDC protocol of realtime RTPCR for influenza A (H1N1). WHO Collaborative Center for Influenza at CDC, Atlanta, GA.
Table 1: Diagnostic Accuracy of DFA and Culture for Diagnosis of the 2009 H1N1 Strain of Influenza A

<table>
<thead>
<tr>
<th></th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
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<tbody>
<tr>
<td></td>
<td>(95% CI)</td>
<td>(95% CI)</td>
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<tr>
<td>DFA</td>
<td>65.0 (59.0, 71.0)</td>
<td>99.6 (99.1, 100)</td>
<td>98.8 (97.0, 100)</td>
<td>85.9 (83.2, 88.7)</td>
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<tr>
<td>Culture</td>
<td>51.8 (40.8, 62.8)</td>
<td>99.6 (99.0, 100)</td>
<td>95.6 (89.3, 100)</td>
<td>92.3 (90.0, 94.6)</td>
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<tr>
<td>DFA+ or Culture</td>
<td>81.3 (76.4, 86.2)</td>
<td>99.2 (98.5, 99.9)</td>
<td>98.0 (96.1, 99.9)</td>
<td>91.9 (89.7, 94.1)</td>
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*aCulture performed only on DFA negative patients.

95% confidence intervals (CI) are presented in parentheses.

Positive predictive value (PPV); negative predictive value (NPV)