Evaluation of the Prodesse Hexaplex Multiplex PCR Assay for Direct Detection of Seven Respiratory Viruses in Clinical Specimens

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**Key Words:** Multiplex PCR; Respiratory viruses; Influenza; Respiratory syncytial viruses; Human parainfluenza viruses

**Abstract**

We evaluated the Hexaplex assay (Prodesse, Waukesha, WI) for the detection of 7 respiratory viruses (influenza A and B, parainfluenza 1-3, and respiratory syncytial virus [RSV] A and B). The Hexaplex assay was performed on 300 respiratory samples during the 1999-2000 respiratory virus season. Results of this assay were compared with shell vial cell culture and/or direct fluorescent antibody stain. The overall sensitivity and specificity of the assay were 96.6% and 94.1%, respectively. The respective sensitivity and specificity of the Hexaplex assay for detection of specific virus groups were as follows: influenza A, 98.6% and 97.8%; influenza B, 100% and 100%; and for parainfluenza viruses (1-3), 100% and 99.1%. The assay did not perform as well with patients infected with RSV: sensitivity and specificity were 91.0% and 98.6%, respectively. There are 2 major drawbacks to this assay: it is technically demanding (3-4 hours hands-on time), and it is expensive ($80-$90 direct cost). Nevertheless, because of the excellent sensitivity and specificity, the Hexaplex assay may be valuable in the diagnosis of respiratory viral infections in immunocompromised patients.

Respiratory infections caused by influenza viruses (A and B), parainfluenza viruses (1, 2, and 3) and respiratory syncytial viruses (RSV; A and B) can result in severe lower respiratory tract infections that may require hospitalization. These viral agents account for about 80% to 90% of viral acute lower respiratory tract infections in infants and for 30% of all childhood deaths in developing countries. The results of these infections can range from mild respiratory illness to primary viral pneumonia and death. Infants, elderly people, and people with underlying health problems are at increased risk for complications that may result in hospitalization.

Rapid differential diagnosis of these agents is necessary for monitoring infected patients, for the prevention of nosocomial spread, and to guide the choice of possible specific antiviral therapy. Moreover, Woo et al demonstrated that rapid viral diagnosis for respiratory infections at a tertiary-care university hospital reduces patients’ hospital stay and costs and reduces antibiotic use by up to 52%.

The use of classic diagnostic techniques such as viral isolation and serologic testing often can result in a delay of several weeks before results are available, and these methods are therefore less useful for making therapeutic decisions. Several immunofluorescent, optical immunoassay, and enzyme-linked immunosorbent assays for direct detection of influenza and parainfluenza virus antigens are available commercially. However, these assays lack the sensitivity (50%-90%) for rapid diagnosis of these viral pathogens. In addition, direct antigen testing also may fail to detect emerging virus variants having altered amino acid sequences on envelope or outer capsid proteins. Finally, specimen integrity and the number of intact cells present in the specimens are crucial for reliable direct immunofluorescence assay results.
Rapid and sensitive molecular diagnostic techniques (reverse transcriptase–polymerase chain reaction [RT-PCR]) for the detection of one or several respiratory viruses in patient samples have been developed.\textsuperscript{2,19-23} These assays are more sensitive than the classic viral culture or viral antigen detection assay.\textsuperscript{1,2,19} Moreover, these assays allow rapid detection of microorganisms in patients infected with 2 or more viruses, which is more difficult by the classic diagnostic techniques.\textsuperscript{24}

We evaluated the Hexaplex multiplex PCR assay (Prodesse, Waukesha, WI) for the direct detection of 7 respiratory viruses in 300 respiratory samples submitted to ARUP Laboratories, Salt Lake City, UT, during the peak of the 1999-2000 respiratory virus season. This assay simultaneously detects influenza viruses A and B, RSV A and B, and parainfluenza viruses 1, 2, and 3.

\section*{Materials and Methods}

\subsection*{Clinical Specimens}

Three hundred samples submitted to ARUP Laboratories from November 30, 1999, through February 29, 2000, were used to evaluate the Hexaplex multiplex PCR assay. Specimens evaluated were submitted for respiratory virus cell culture, direct fluorescent antibody staining (DFA), or both. Specimen types included 142 nasopharyngeal swabs (47.3\%), 57 nasal washes (19.0\%), 46 throat swabs (15.3\%), 34 bronchoalveolar lavage specimens (11.3\%), 14 sputum (4.7\%), 6 lung tissue (2.0\%) and 1 pleural fluid (0.3\%). Of the specimens, 84.0\% (n = 252) were received in M4 transport medium (Micro Test, Lilburn, GA) at 4°C, while the remaining samples (n = 48; 16.0\%) were collected in sterile containers and also transported at 4°C. Patients ranged in age from 1 week to 99 years. Of the samples, 46.0\% (n = 138) were from male patients and 54.0\% (n=162) were from female patients.

\subsection*{Cell Lines}

All cell lines used in the study were grown in shell vials. Primary rhesus monkey kidney (PRMK) cells were purchased from ViroMed Laboratories (Minneapolis, MN), and buffalo green monkey kidney (BGM) cells, rhabdomyosarcoma (RD) cells, human lung carcinoma cells (A549), and human diploid fibroblast cells (MRC-5) were obtained from the ARUP Cell Culture Laboratory (Salt Lake City, UT). Cells were maintained in Eagle minimal essential medium supplemented with 10\% fetal bovine serum at 37°C in a carbon dioxide incubator. Once inoculated, the cells were maintained in Eagle minimal essential medium supplemented with 2\% fetal bovine serum, 50 U/mL of penicillin, 50 µg/mL of streptomycin, 20 µg/mL of vancomycin, 20 µg/mL of gentamicin, and 1µg/mL of amphotericin B, in a 5\% carbon dioxide incubator at 34°C.

\subsection*{Viral Culture}

Viral culture using shell vial techniques was performed as described by Engler and Selepak\textsuperscript{25} with some modifications that were validated at ARUP Laboratories. Briefly, specimens submitted for influenza virus culture only were inoculated into 3 vials of PRMK cells, while specimens submitted for respiratory virus cultures were inoculated into the following shell vials: 3 with PRMK cells, 1 with BGM cells, 1 with RD cells, 1 with MRC-5 cells, and 1 with A549 cells. Cell monolayers were inoculated and centrifuged at 20°C for 15 minutes at 2,800g.\textsuperscript{25,26} After centrifugation, the vials were incubated in a carbon dioxide incubator at 34°C. The cell monolayers were observed every other day for the presence or absence of cytopathic effects. In addition, the PRMK cells were hemadsorbed on days 3, 6, and 14. All hemadsorption-positive samples were tested for influenza viruses A and B; parainfluenza viruses 1, 2, and 3; RSV; and adenovirus using Bartels fluorescent antibody stain (Bartels, Issaquah, WA). Respiratory cultures were kept a total of 14 days.

\subsection*{DFA Stain}

Bartels Viral Respiratory Screening and Identification Kit, a fluorescent antibody staining method, was performed as directed by the manufacturer with some modifications. Briefly, respiratory samples were mixed thoroughly by a vortex, and a 1- to 2-mL aliquot was poured into a holding tube. Approximately 3 mL of phosphate-buffered saline (PBS) was added to the specimen and centrifuged at 500x for 10 minutes. The supernatant was decanted, and a second wash was performed. After the cell pellet was resuspended in 2 to 8 drops of PBS, cells were spotted onto an acetone-cleaned 8-well slide and allowed to air dry in a biohazard hood. The slides then were fixed with acetone and allowed to dry completely. Adequate amounts of mouse anti-influenza A and B; parainfluenza viruses 1, 2, and 3; and RSV antibodies were added to the appropriate wells. The 8-well slide was incubated with polyclonal respiratory viral pool. The slides were incubated at 37°C for 30 minutes in a humid chamber. After the primary antibody was rinsed with PBS, secondary antibody (antimouse IgG-FITC [fluorescein isothiocyanate]) was added to each well on the slide and incubated for 30 minutes at 37°C. Samples submitted for RSV testing only were processed as for DFA and stained with Bartels single-step RSV antibody for 30 minutes at 37°C. After the antibody was washed off and a cover glass was placed over the sample wells, fluorescent staining was observed using a fluorescence microscope at ×250 and ×400 magnification.

\subsection*{Hexaplex Assay}

The assay was performed as recommended by the manufacturer.\textsuperscript{2} The manufacturer provides 2 PCR protocols...
to run the assay, one with enzyme contamination control (uracil N-glycosylase [UNG] protection) and one with no UNG protection. We evaluated the PCR protocol with UNG protection since UNG contamination control is the standard procedure in our laboratory.

Briefly, clinical samples received in the clinical virology laboratory in M4 viral transport media or in sterile containers at 4°C were mixed and processed by viral cell culture, DFA staining, or both. A 0.5- to 1.0-mL aliquot also was transferred to a sterile tube and stored at −70°C for later evaluation by the Hexaplex assay. All frozen samples were tested within 1 month of storage. Patient samples were thawed once on ice, centrifuged at 1,000g for 10 minutes at 4°C to remove cellular debris, and placed on ice. Viral genomic RNA was extracted from patients’ sample supernatants using the QIAamp (Qiagen, Valencia, CA) RNA extraction kit. Viral RNA was transcribed to complementary DNA using Reverse Transcription Mixture (Prodesse), magnesium chloride (PE Applied Biosystems, Foster City CA), RNase Inhibitor (PE Applied Biosystems), and MuLV reverse transcription enzyme (PE Applied Biosystems). PCR amplification of viral complementary DNA was performed by using Hexaplex Super-Mix (Prodesse) and AmpliTaq Gold DNA polymerase (PE Applied Biosystems). For contamination control, AmpErase Uracil N-glycosylase (PE Applied Biosystems) and dUTP (deoxyuridine triphosphate; PE Applied Biosystems) were used. After amplification, the PCR product was purified with the QIAquick PCR purification kit (Qiagen), heat denatured, and added to NeutrAvidin-coated strip plates (Prodesse). Denatured DNA products were detected by peroxidase-labeled probe for each of the 7 viruses. After a capture and hybridization reaction, substrate solution was added to each well. The reaction was stopped with stop solution, and the optical density (OD) of each well was measured at 450 nm in a spectrophotometer. Previously reported positive patient specimens were used as positive controls and water as a negative control as recommended by the manufacturer. We further used detection controls (for each of the 7 viruses, PCR products from patients with positive results) to evaluate probe performance on each run.

Sensitivity Studies

Viral RNA transcripts provided in the Hexaplex assay kit were used to evaluate the analytical sensitivity of the Hexaplex assay. RNA transcripts (5 × 10^5 copies/µL), for each of the 7 viruses supplied by the manufacturer, were serially diluted in ribonuclease (RNase) free water supplied by the manufacturer and supplemented with RNase Inhibitor (PE Applied Biosystems). The samples were then evaluated by the Hexaplex assay from the RNA extraction step as recommended by the manufacturer.

Hexaplex Cross-Reactivity

The cross-reactivity of the Hexaplex assay with different viruses or bacteria was determined by using recovered patient isolates. The Hexaplex assay did not cross-react with the following isolates: adenovirus, 5; cytomegalovirus, 6; herpes simplex virus, 4; enteroviruses, 5 (echovirus serotype 9, 3; coxsackie virus B5, 2); Chlamydia pneumoniae, 1; and Mycoplasma pneumoniae, 1.

Data Interpretation

An OD of 0.4 or more was used as the cutoff for positive samples as indicated by the manufacturer. Patient samples that had an OD between 0.35 and 0.40 were reextracted and reprocessed with the Hexaplex assay, and if the reading was still in that range, the samples were called negative. For discrepant results between PCR and culture/DFA, both methods were repeated.

Results

Hexaplex Analytical Sensitivity

Viral RNA transcripts of known concentrations provided by the manufacturer were serially diluted to determine the analytical sensitivity of the Hexaplex assay. We report the sensitivity of the assay based on the assumption that 100% of the transcripts put in the RNA extraction procedure were recovered. The sensitivities of the viruses were as follows: influenza A, 42 copies; influenza B, 42 copies; parainfluenza virus 1, 4,200 copies; parainfluenza virus 2, 42 copies; parainfluenza virus 3, 42 copies; RSV A, 4,200 copies; and RSV B, 42 copies.

Overall Performance of the Hexaplex Assay Compared With Cell Culture and DFA

A total of 148 of 300 specimens were positive for one of the respiratory viruses by either DFA or culture. Thus, the positivity rate of the specimens evaluated by the 3 methods during the peak 1999-2000 respiratory season was 49.3%. Of the 148 specimens, 143 were positive by the Hexaplex assay, for an overall sensitivity of 96.6%. Nine samples were positive by the Hexaplex assay alone. These results were not reported as positive; thus, the overall specificity of the assay was 94.1%. The positive predictive value (PPV) and the negative predictive value (NPV) for the Hexaplex assay were 94.1% and 96.6%, respectively.

Hexaplex Detection of Influenza Viruses

The ability of the Hexaplex assay to detect influenza viruses (A and B) was evaluated on 254 respiratory samples submitted for cell culture, DFA, or both. We cultured 159
samples and tested 211 by the DFA procedure. Of the 75 patient samples positive by either DFA or cell culture, 74 were positive for influenza A virus by the Hexaplex assay (98.6% sensitivity). Of the 179 samples negative by the DFA and/or cell culture, 4 were positive by the Hexaplex assay (97.8% specificity). The results for only 1 patient during the study period were positive for influenza B by cell culture. This patient’s sample was also positive for influenza B by the Hexaplex assay.

Compared with cell culture alone, the Hexaplex assay had sensitivity and specificity of 97.4% and 99.1%, respectively (Table 1), while the PPV and NPV were 92.5% and 99.4%, respectively. When time to detection of influenza virus by cell culture was analyzed for the 38 positive specimens, 58% (n = 22) were detected after 3 days of incubation, 32% (n = 12) were detected after 6 days of incubation, and 11% (n = 4) were detected after 14 days of incubation.

Compared with DFA alone the Hexaplex assay had sensitivity and specificity of 100% and 98.6%, respectively (Table 1), while the PPV and NPV were 97% and 100%, respectively. When the time to detection by cell culture was evaluated, 27% (n = 3) of the 11 positive samples were detected on day 3, 55% (n = 6) were detected on day 6, and 18% (n = 2) were detected on day 14 of incubation.

Compared with DFA alone, the Hexaplex assay had sensitivity and specificity of 100% and 96.3%, respectively (Table 1), while the PPV and the NPV were 73% and 100%, respectively. Six of the 8 samples negative by DFA but positive by the Hexaplex assay were positive by cell culture. By using culture for discrepant analysis compared with DFA, the resolved specificity is 99%, and the PPV is 93%.

**Hexaplex Detection of Parainfluenza Viruses 1, 2, and 3**

During the 1999-2000 respiratory season, 262 patient samples were evaluated by cell culture, DFA, or both for infection with the parainfluenza virus (1, 2, or 3). We cultured 158 samples and tested 243 by the DFA. Of these samples, 29 positive samples by DFA and/or cell culture also were positive by the Hexaplex assay (sensitivity, 100%). Nineteen patient samples (66%) had parainfluenza virus 1, 4 samples (14%) had parainfluenza virus 2, and 6 samples (21%) had parainfluenza virus 3. Two samples were negative by both DFA and cell culture but positive by the Hexaplex assay for either parainfluenza virus 1 or 2 (specificity, 99.1%) (Table 1). The PPV and NPV were 94% and 100%, respectively.

Based on positive samples by cell culture alone, the Hexaplex assay had sensitivity and specificity of 100% and 98.6%, respectively (Table 1), while the PPV and the NPV were 85% and 100%, respectively. When the time to detection by cell culture was evaluated, 27% (n = 3) of the 11 positive samples were detected on day 3, 55% (n = 6) were detected on day 6, and 18% (n = 2) were detected on day 14 of incubation.

Compared with DFA alone, the sensitivity and specificity of the Hexaplex assay were 100% and 96.3%, respectively (Table 1), while the PPV and the NPV were 73% and 100%, respectively. Six of the 8 samples negative by DFA but positive by the Hexaplex assay were positive by cell culture. By using culture for discrepant analysis compared with DFA, the resolved specificity is 99%, and the PPV is 93%.

**Table 1**

Comparison of Hexaplex Assay Performance With Detection of the Virus Groups With Cell Culture and/or Direct Fluorescent Antibody Staining (DFA)

<table>
<thead>
<tr>
<th>Hexaplex</th>
<th>Culture/DFA</th>
<th>Culture</th>
<th>DFA</th>
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<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
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<tr>
<td>Influenza A virus</td>
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<tr>
<td>Positive</td>
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<td>4</td>
<td>37</td>
</tr>
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<td>Negative</td>
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<td>1</td>
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<td>Specificity (%)</td>
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<tr>
<td>Parainfluenza virus</td>
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<td>0</td>
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<td>Sensitivity (%)</td>
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<td>100</td>
<td>100</td>
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<td>Specificity (%)</td>
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<tr>
<td>Positive</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Negative</td>
<td>ND</td>
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<td>ND</td>
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<tr>
<td>Sensitivity (%)</td>
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<tr>
<td>Specificity (%)</td>
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</table>

ND, not done.

* Prodesse, Waukesha, WI.
The high sensitivity and specificity of the Hexaplex assay are consistent with the analytical sensitivity of the assay. Serial dilutions of RNA transcripts provided by the manufacturer revealed a detection limit ranging from 42 copies to 4,200 copies. These results are higher than those reported by the manufacturer (5-10 copies). This could be due to the manufacturer not taking the RNA transcripts through the RNA extraction procedure.2 The Hexaplex assay’s analytical sensitivity should permit the detection of the microorganism in the majority of infected patients since, on average, early in infection, patients shed 3 to 7 log10/mL influenza viruses, 4 to 5 log10/mL parainfluenza viruses, and 5 to 7 log10/mL of RSV.30-32

During the 1999-2000 influenza virus season at ARUP Laboratories 99% (n = 75) of the influenza viruses isolated were influenza virus A, and there was only 1 (1%) influenza B virus recovered. These results are consistent with the surveillance studies done by the Centers for Disease Control and Prevention, which indicated that the 99.8% of influenza viruses isolated were influenza A virus and 0.2% were influenza B virus.33 The Hexaplex assay’s sensitivity and specificity for detecting influenza A virus were similar to the overall sensitivity and specificity of the assay. The assay missed influenza A virus in 1 patient sample that was positive by cell culture. Repeated testing indicated that this was a false-negative result. On the other hand, the Hexaplex assay detected 4 samples with influenza A virus that were negative by culture and DFA. The clinical histories on the 4 patients indicated respiratory disease compatible with influenza. Similar to cell culture, the Hexaplex assay detected the only influenza B virus from 1 of the patient samples.

The Hexaplex assay’s sensitivity and specificity for detecting parainfluenza viruses 1, 2, and 3 were excellent. The assay had an overall 100% sensitivity and 99.1% specificity. Two patients were positive by the Hexaplex assay for parainfluenza virus (1 or 2) but negative by DFA and/or culture. Retesting the patient samples by DFA was also negative for these 2 viruses. Clinically, the 2 patients had respiratory symptoms indicating an infection.

In our study, the sensitivity of the Hexaplex assay for detecting RSV viruses (91%) was less than that reported by the manufacturer (99.8%). Of the 44 samples reported positive by DFA, 82% (n = 36) came from patients younger than 2 years, and 18% (n = 8) were from patients older than 2 years. On the other hand, the specificity of the Hexaplex assay was 98.6%, similar to that reported by the manufacturer (97.6%). The Hexaplex assay missed microorganisms in 4 patients whose results were repeatedly positive by DFA. These 4 samples were reported positive based on the typical RSV cytoplasmic staining by DFA. Several groups have reported higher RSV analytical sensitivity (96%-97%) using a different multiplex assay.1,7,34 One explanation for this may
be the higher detection limit (4,200 copies) of the Hexaplex assay for detecting RSV A RNA transcripts. In the present study, we did not type the 4 isolates missed by the Hexaplex assay. The 4 samples also could have been missed owing to genetic variations in the RSV genome. Some of these genetic variations have been postulated to have a role in the ability of RSV to establish reinfections throughout life.

Multiplex testing also permits detection of viruses not suspected of causing the disease. In the present study, 3 patient samples (1.2% of total samples submitted for RSV testing) submitted for RSV testing by DFA were reported negative by DFA but actually were positive for parainfluenza virus 1. These viral infections can have similar clinical manifestations; therefore, if testing is ordered for a specific respiratory virus, other pathogens may be missed.

As suggested, one of the potential advantages of using a multiplex assay is its ability to detect dual infection. Currently, the reported frequency of dual respiratory viral infections varies widely, and the importance of such infection is unresolved. The detection of dual infection depends on how many diagnostic techniques are used. In the study by Drews et al, when cell culture alone was used as the sole diagnostic technique, the rate of dual respiratory viral infections was 1.9%. When culture, serologic testing, and PCR were used, the dual infection rate was 11.6%.

If the results of PCR are presumed to be correct, then in our study, the rate of dual infection was 1.3%, as 4 patients had 2 different respiratory viruses detected by all 3 methods. For the first patient, influenza A virus and parainfluenza virus 2 virus were detected in the respiratory sample. However, culture and DFA detected only influenza A virus. For 2 other patients with dual infections, the samples had influenza A virus and RSV, both of which also were detected by DFA. It is notable that we had 1 patient sample that had both adenovirus and RSV. In our laboratory, the adenovirus overgrew the RSV virus; thus, RSV was not reported as present. However, the Hexaplex assay detected the RSV virus even in the presence of adenovirus. Using the multiplex assay may help to resolve the frequency and clinical importance as well as the severity of dual infections.

A rapid molecular diagnostic technique ideally should be cost-effective and easy to perform. The Hexaplex assay does not meet these 2 criteria. The direct costs (manufacturer’s kit and reagents) for running the Hexaplex assay are in the range of $80 to $90. This does not include the royalties that must be paid to Roche Diagnostics (Indianapolis, IN) for running the PCR assay or for other supplies and technologist time. For this reason, the assay should be reserved for diagnosing viral respiratory infections in severely ill patients or patients with complicated conditions (immunocompromised patients and transplant patients). We do not believe that it is cost-effective to perform the assay on immunocompetent outpatients suspected of having a viral infection. At ARUP Laboratories, estimated direct costs for viral culture and for the respiratory virus DFA panel are $13 and $15, respectively.

The Hexaplex assay is a labor-intensive assay. It is estimated to take 8 to 9 hours to complete the assay with about 3 to 4 hours of hands-on time depending on the expertise of the technologist. Hands-on times for cell culture and DFA were estimated to be 15 minutes and 10 minutes, respectively.

The Hexaplex assay is a sensitive assay for detecting 7 of the most common respiratory viruses that account for up to 80% of all viral respiratory infections. The main drawbacks of this assay are its cost and its labor-intensive nature.

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References


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